CHARACTERIZING THE MOLECULAR MECHANISMS OF BLOOD PRESSURE REGULATION BY GRAF3, A SMOOTH MUSCLE-SPECIFIC RHO-GAP

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pathology and Laboratory Medicine in the School of Medicine.

Chapel Hill
2019

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ABSTRACT

Rachel Ann Dee: Characterizing the molecular mechanisms of blood pressure regulation by GRAF3, a smooth muscle-specific Rho-GAP
(Under the direction of Joan Taylor)

Although hypertension (HTN) is a major risk factor for stroke, myocardial infarction, and kidney failure and contributes to over 350,000 deaths annually in the United States, we know surprisingly little about its development or the mechanisms by which it promotes cardiovascular disease (CVD). Blood pressure (BP) is complex and tightly regulated by the integrated control of multiple organs including the brain, heart, kidneys, GI tract, endocrine and vascular systems. A key feature of HTN is increased peripheral vascular resistance which is controlled primarily by vascular smooth muscle cell (SMC) contractility. We identified a SMC-specific RhoA-GAP (termed GRAF3) that is critical for limiting RhoA dependent SMC contractility and for controlling BP homeostasis. The work presented below aimed to assess the therapeutic potential of GRAF3 and to identify its mechanisms of biochemical regulation.

Excitingly, to this end, we found that SMC overexpression of GRAF3 resulted in a significant 10 mmHg reduction in basal systolic BP—a reduction that was maintained throughout L-NAME induced HTN. Interestingly, based on molecular modeling and *in vitro* experiments, we found that the GAP activity of GRAF3 is inhibited by an auto-regulatory interaction between the GRAF3 BAR-PH domains and the GRAF3 GAP domain. Furthermore, we used our models to predict important sites of post-translational modifications. We were able to show that Y367 is the major site of GRAF3 activation and phospho-mimetic variants show enhanced GAP activity

and decreased RhoA activity, indicating the importance of this site for GRAF3 functionality. Moreover, we showed that Src and FAK kinases phosphorylate GRAF3 at Y376 as part of a mechanotransduction feedback loop.

During this project, we discovered the potential importance of another seemingly SM-specific RhoGAP, GRAF2. We set out to discover whether the role of GRAF2 overlaps that of GRAF3 and found that not only does GRAF2 depletion lead to an increase in basal BP, but it also upregulates SMC gene expression and may play a role in arterial stiffening, another feature common to CVD. Collectively, the work presented in this dissertation may aid in the development of novel anti-hypertensive therapies.

To my parents, Tim and Gayle, for their unconditional support and unwavering confidence in me. And to Matt, for always reminding me, “you need to relax”.

ACKNOWLEDGEMENTS

There are so many people that have been instrumental to my Ph.D. training. First and foremost, I want to thank my PI and mentor, Joan Taylor, for being the best role model I could have asked for at UNC. Joan's excitement for our science is infectious and I walked out of every meeting with her more energized than when I walked in. Thank you for helping me stay on track whenever I felt lost and for pushing me to improve my weaknesses without ever making me feel bad about them. The last year of my Ph.D. has been really tough for me, but you were always in my corner and never stopped having faith in me. Thank you for always putting the human before the science. I was thankful from the start to have joined your lab, but every year I grew more thankful than the last. You have been influential not only to my development as a scientist, but also to my development as a supervisor. Whenever I was in a situation where I didn't quite know what to do, I'd stop and ask myself, "What would Joan do?", and there would be the answer. There are no words strong enough to express my gratitude and appreciation.

I feel very lucky to have a 2nd (unofficial) PI, Chris Mack, to mentor me through graduate school. Thank you for always bringing a little healthy skepticism to lab meeting and asking insightful questions about my experiments. And thank you for always making me feel like you trusted and valued my answers to those questions! Thank you for being the best sports bracket commissioner and doing the numerous other little things you do to make our lab such an enjoyable place. All these small gestures—like giving me a running themed AHA pin from a

conference you attended, just because you thought I'd like it—make a huge difference and set you apart from other mentors.

I would also like to thank the additional members of my committee, Bill Arendshorst, Anthony Viera, and David Williams, for their vital input and suggestions to make my project the best that it could be. Thank you for making me feel like my committee meetings were just friendly conversations.

I would like to thank all the past and present members of the Taylor/Mack labs, especially Xue Bai, Qiang Zhu, Zachary Opheim, and Matthew Combs. Whether we talked about science, politics, pop-culture, or life, I have always enjoyed your company and comradery. I genuinely feel invested in one another's success and will always cherish how we help bring each other up to the next level. I'm so thankful to have shared an office with you all and that we never had to choose someone to move to the "other room". Thank you to Kevin Mangum, for your encouraging nature and for always taking the time to talk and give me sound advice.

I'd like to give a very special extended thank you to Xue Bai, for the tremendous amount of mentoring she gave me. I wouldn't be half the scientist I am without your teaching and teamwork. Thank you for setting the foundation of this project and for your scientific contributions to Chapters 2 and 3 of this dissertation. Thank you for always being patient with me and letting me ask you "one more question" several times over.

This work wouldn't have been possible without the help of several outstanding people at UNC Core Facilities. Pablo Ariel, Victoria (Vicky) Madden , and Kristen White at the Microscopy Services Laboratory. Thank you for reteaching me how to use the microscope every time I touched it. And thank you, Pablo, for going to such great lengths to improve my methods and analyses. Thank you to Brian Cooley at the McAlister Heart Institute Animal Surgery Core.

Not only for your surgical expertise, but for your riddles and stimulating conversation, too. Thank you as well, to Dale Cowley and the Animal Models core and Brenda Temple at the Structural Bioinformatics Core.

I would be remiss to not give thanks to the American Heart Association, the National Institutes of Health, the UNC Department of Pathology and Laboratory Medicine, and the UNC Graduate School Royster Society of Fellows for funding me and our research.

A huge thank you is in order to all the wonderful staff in the Training Initiatives in Biological and Biomedical Sciences (TIBBS) office, for all the activity planning, skill building workshops, and career preparation seminars they do. This unique program is one of the reasons I chose UNC for graduate school. I'd like to especially thank Rebekah (Beka) Layton, Director of TIBBS, for being my career coach and life coach and for always holding me accountable.

Last but not least, thank you to all the friends and family without whom this would never have been possible. Grad school can be very stressful, and you all are the real reason I made it through. To my "Garter Girls", "Run Squad", and "Bachelorette Gang": This is just the beginning of a life-long friendship. I love seeing each of us grow personally and professionally and whole-heartedly support one-another. To my siblings, Adam (Jenni, Raelynn), Sarah (Ryan, Brooke), and Matthew for standing by my side no matter what. And for always being down to play board games together when I come home. You guys don't know how much I brag about you to other people! Thank you to Matt, for always being ready for an adventure and for keeping me level-headed. Thank you to my parents, Tim and Gayle, for absolutely everything. For always supporting my dreams, no matter what (or where!) they are. And for making me feel loved every day of my life. I hope to always make you proud.

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LIST OF ABBREVIATIONS

ACE	angiotensin converting enzyme
AII	angiotensin II
BAR	Bin/amphiphysin/Rvs
BFA	Brefeldin A
BP	blood pressure
CNS	central nervous system
CVD	cardiovascular disease
DOCA	deoxycorticosterone-acetate
ENaCs	epithelial sodium channels
ERM	ezrin-radixin-moesin
ET1	endothelin-1
FAK	focal adhesion kinase
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
GRAF	GTPase Regulator Associated with Focal adhesion kinase
GTP	guanosine triphosphate
GWAS	genome-wide association studies
HTN	hypertension
HuAoSMC	human aortic smooth muscle cells

L-NAME	L-N^G-Nitroarginine methyl ester
MD	macula densa
mDia	mammalian Diaphanous
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MRTF-A	myocardin related transcription factor-A
MRTF-B	myocardin related transcription factor-B
NHE3	sodium-hydrogen exchanger
NTS	nucleus tractus solitarii
PAH	pulmonary arterial hypertension
PE	phenylephrine
PH	pleckstrin homology
pMLC	phosphorylated myosin light chain
RaAoSMC	rat aortic smooth muscle cells
ROCK	Rho Kinase or Rho-associated coiled-coil domain containing protein kinases
S1P	sphingosine-1-phosphate
SH3	SRC Homology 3
SHR	spontaneously hypertensive rat
SM	smooth muscle
SMC	smooth muscle cell
SRF	serum response factor

CHAPTER 1: INTRODUCTION[1]

Although hypertension (HTN) is a major risk factor for stroke, myocardial infarction, and kidney failure and contributes to over 350,000 deaths annually in the United States[1], we know surprisingly little about its development or the mechanisms by which it promotes cardiovascular disease (CVD). A number of antihypertensive drugs are available, but regimens are usually chosen empirically and multiple drugs that target different organ systems are frequently required for effective treatment. One reason for these difficulties is that blood pressure (BP) is a complex trait that is regulated by many organ systems and a large number of humoral factors. Thus, a better understanding of the molecular and genetic mechanisms that control BP under normal and pathologic conditions should lead to novel drug targets and/or to personalized therapies that are more effective and less toxic. Recent advances suggest that RhoA signaling plays a role in the development of human HTN. The focus of this introduction will be 1) to provide a very brief overview of HTN, 2) to highlight the mechanisms underlying RhoA-dependent regulation of BP, 3) to summarize the current data on the strategies and efficacy of targeting this pathway in hypertensive patients, and 4) to state the objectives of this dissertation.

[1] This chapter contains text and figures previously published in the following review articles:
Bai X, Dee R, Mangum K, Mack C and Taylor J. RhoA signaling and blood pressure: The consequence of failing to Tone it Down". *World J Hypertens*. 2016;6:18-35.
Dee RA, Mangum KD, Bai X, Mack CP and Taylor JM. Druggable targets in the Rho pathway and their promise for therapeutic control of blood pressure. *Pharmacol Ther*. 2019;193:121-134.

1.1 A brief overview of hypertension

Hypertension is a major cardiovascular risk factor that significantly increases the incidence of stroke, myocardial infarction, heart failure, retinopathy, and kidney disease[2]. Although HTN is one of the most modifiable cardiovascular risk factors, the number of individuals with HTN is increasing world-wide. Further amplifying the importance of HTN, the American Heart Association has recently revised its definition of Stage 1 HTN to include individuals with systolic BP between 130 and 139 mmHg or diastolic BP between 80 and 89 mmHg. This change was prompted by studies demonstrating beneficial effects of lowering BP below the 120/80 mmHg threshold[3-5] and effectively increased the number of Americans categorized as hypertensive from 32% to 46%[5]. It is also becoming clear that many people suffer from masked HTN (normal readings in the clinic, but hypertensive outside the clinic) and non-dipping HTN (steady BP through the day but no decrease in BP at night)[6-8], suggesting that more intensive BP monitoring would identify additional at-risk individuals[9].

A number of relatively inexpensive first-line therapies are available to treat HTN including diuretics, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II (AII) receptor blockers, and calcium channel blockers. However, these drugs are usually prescribed empirically and are often ineffective. Indeed, over 50% of adults who are being treated for HTN still do not have their BP under control[10]. Although treatment can be improved by multidrug regimens that target different BP control mechanisms, 13% of treated patients have drug-resistant HTN and remain hypertensive even after taking three 3 or more medications, or require 4 medications for adequate BP control[11, 12]. Taking multiple BP medications also increases the risk of unwanted side effects and drug-drug interactions. While incomplete health history and

poor patient compliance contribute to the difficulties in treating HTN, our lack of understanding of the etiology of HTN is also a major factor.

Many of the difficulties of treating HTN stem from the fact that BP is an extremely complex trait regulated by many organ systems. Although the major determinants of BP are cardiac output and systemic vascular resistance, BP homeostasis requires proper regulation of heart and vasculature function by the autonomic nervous system, kidneys, and endocrine organs. The fact that these systems are tightly integrated by many feedback loops further complicates our understanding of the development of HTN and its treatment. Nearly all heritable genetic mutations that cause HTN affect kidney function and/or salt balance, but these variants only explain about 10% of HTN cases. More recent genome wide association studies (GWAS) have identified many genetic loci that correlate with relatively small differences in BP between populations. However, because most of these variations are within or near genes with no known connection to BP regulation, our understanding of how they affect the development of HTN is limited. A number of the genes identified by GWAS are highly expressed in endothelial and smooth muscle cells (SMCs), highlighting the importance of the vasculature as a major regulator of BP and as a target for potential therapies[13-16].

1.2 Impact of the RhoA pathway on blood pressure homeostasis

The 22 members of the Rho family of small GTPases can be divided into three major subfamilies: Rac, RhoA, and Cdc42. The RhoA family of GTPases (RhoA, RhoB, and RhoC) are widely expressed and share 85% amino acid homology including a C-terminal cysteine residue that is the target of geranylgeranylation, a post-translational modification that anchors RhoA family proteins to the plasma membrane. As discussed below, RhoA is by far the most studied

member of this subfamily and has been shown to regulate a variety of cellular processes including (but not limited to) actin and microtubule dynamics, cell force, cell shape and polarity, endocytosis, exocytosis, cell adhesion and migration, proliferation, and differentiation[17, 18].

Like all GTPases, RhoA is regulated by guanosine triphosphate (GTP) binding and cycles between the active GTP-bound form and the inactive guanosine diphosphate (GDP)-bound form and this cycle is under the direct control of three groups of regulatory protein; guanine dissociation inhibitors (GDIs) sequester RhoA into an inactive cytoplasmic fraction, guanine nucleotide exchange factors (GEFs) activate RhoA by facilitating exchange of GDP for GTP, and GTPase activating proteins (GAPs) promote RhoA's intrinsic GTPase activity to hydrolyze GTP to GDP and efficiently turn off (or limit) RhoA-dependent signaling. When GTP-bound, RhoA interacts with a variety of effector molecules that mediate its varied functions including the Rho-associated coiled-coil domain containing protein kinases (ROCK I and II), the diaphanous-related formins (mDia1 and mDia2), protein kinase N, citron kinase, rhophilin, and the rhotekins I and II, among other enzymes[17].

Recent advancements suggest that RhoA signaling in the vasculature is a particularly attractive target for therapeutic intervention in the treatment of HTN. With respect to regulation of BP, Rho kinases are arguably the most important effectors as evidenced by the findings that increased ROCK activity has been observed in spontaneously hypertensive rats and some hypertensive patient populations[19, 20] and that ROCK inhibitors like Y-27632, Fasudil, and SAR407899 have been shown to reduce BP in hypertensive animal models and patients[21]. Extensive studies have shown RhoA signaling enhances Ca^{2+}-dependent, myosin-based force production in vascular SMCs and recent studies from our lab and others have implicated several components of the RhoA signaling pathway in the development of HTN in mouse models[19, 20, 22].

Moreover, human genetic studies have identified BP-associated variants in several additional Rho-related genes further implicating this pathway in human HTN[23].

Although Rho signaling components are relatively strongly expressed in vascular SMCs, nearly all, with the exception of the RhoGAP GRAF3, are expressed in many other tissues. Thus, when evaluating Rho signaling molecules as targets of anti-HTN therapy, it is important to consider the potential impact of modulating Rho-signaling in other organ systems. Interestingly, with respect to BP regulation, studies using pre-clinical models indicate that attenuating RhoA signaling in the vasculature, kidney, myocardium, and CNS could all lead to the desired outcome of lowering BP.

1.2.1 RhoA and arteriole tone

Vascular resistance is a major determinant of BP and is controlled, in large part, by smooth muscle cell contraction within small peripheral arterioles[24-28]. Mechanistically, excitation-contraction coupling in SMCs is mediated by the Ca^{2+}-dependent activation of myosin light chain kinase (MLCK), and SMC tension is directly proportional to myosin light chain (MLC) phosphorylation at S19 as this enables myosin's molecular interaction with actin[29, 30] (**Figure 1.1**). Interestingly, besides promoting an inositol triphosphate-mediated increase in intracellular Ca^{2+}, many circulating GPCR-coupled contractile agonists including AII, norepinephrine, endothelin-1 (ET1), and sphingosine-1-phosphate (S1P) also stimulate RhoA activity in SMCs and in intact arteries which further enhances Ca^{2+}-dependent SMC contractility[19, 20, 22]. Active RhoA leads to ROCK-dependent inhibition of myosin phosphatase and results in elevated MLCK activity and enhanced sensitization to Ca^{2+} [19, 31-33]. Importantly, several studies in animal models and patients (described in further detail below) indicate that

RhoA-dependent pathways are involved in the increased vascular resistance associated with hypertension[19-22, 34].

Studies in genetically engineered mice revealed that germline deletion of the Rho-specific GEF, LARG, significantly attenuated salt-induced HTN[35], while SMC-specific knockout of the related GEF, p115RhoGEF, inhibited the development of HTN in response to AII[20]. In addition, we recently showed that depletion of the SMC-selective, Rho-specific GAP, GRAF3 (ArhGAP42) in mice leads to basal HTN, increased pressor responses to AII, ET1, and phenylephrine (PE), and elevated deoxycorticosterone-acetate (DOCA)-salt induced HTN[36-38].

RhoA regulates several effector molecules that impact SMC contractility (**Figure 1.1**). Direct phosphorylation of MLC by ROCKs I and II promotes actin-myosin crossbridge cycling as does ROCK dependent inhibition of MLC phosphatase (MLCP). RhoA activity is also critical for *de novo* formation of actin filaments and formation of focal adhesions that are required for myosin-dependent force development and transmission, respectively. The Rho effectors mDia 1 and 2 are the most potent regulators of actin filament formation as these proteins function to directly catalyze actin polymerization in cooperation with the actin binding protein, profilin. ROCKs also inhibit actin de-polymerization by phosphorylating and activating LIM-kinase 1 and 2 (on Thr 508 or 505 respectively), which in turn, phosphorylate and inhibit the actin filament severing protein, cofilin[39-42]. Finally, ROCK-dependent phosphorylation of ezrin-radixin-moesin (ERM) proteins promotes their tethering to integral plasma membrane proteins effectively stabilizing actin filaments and increasing force transmission[43].

In addition to promoting acute changes in SMC contractility, recent studies indicate that RhoA signaling also controls the transcription of numerous contractile genes by modulating serum response factor (SRF) activity. SRF binds to CArG ($CC(A/T)_6GG$) cis elements located

within the promoters of nearly all SMC contractile genes (including SM myosin heavy chain, SM22, calponin, and SM α-actin)[44]. SRF activity is modulated by transcription cofactors of the myocardin family[45-49] and two such co-factors, myocardin transcription factor A and B (MRTF-A and MRTF-B) mediate strong trans-activation of SMC contractile genes[50, 51]. We have previously demonstrated that RhoA promotes SMC contractile gene expression through actin polymerization-dependent regulation of MRTF-A and MRTF-B nuclear localization[44, 51-54]. Cytoplasmic monomeric G-actin is abundant when RhoA activity is low (for example in SMC under low tension[55]), and under these conditions, G-actin binds to MRTF and masks an N-terminal nuclear localization sequence, resulting in cytoplasmic sequestration of these SRF co-factors. Upon RhoA activation, G-actin is recruited into growing F-actin filaments and MRTF-G-actin association decreases. As a consequence, MRTF nuclear localization sequence is un-masked, and MRTF accumulates in the nucleus and promotes SRF-dependent gene expression[56]. Moreover, elevated RhoA in endothelial cells impairs endothelial cell-mediated vasorelaxation as it decreases availability of the potent vasodilator, nitric oxide by reducing both eNOS expression and activity[57-61]. Thus, signaling through RhoA in small arteriolar SMC enhances Ca^{2+} sensitivity, promotes actin remodeling and induces expression of contractile proteins and these responses are necessary for maintaining sustained SMC contractility and elevated vessel tone.

1.2.2 RhoA and kidney function

The kidneys play a major role in regulating BP by controlling sodium excretion and blood volume. In addition, since the kidneys are highly perfused organs receiving up to 25% of total cardiac output, increased contractility of renal arterioles can significantly increase total

peripheral vascular resistance. In most vascular beds, arteriolar tone is controlled by autonomic innervation and circulating hormones. However, in pre-glomerular afferent arterioles, increased kidney perfusion (manifesting as increased renal BP) stimulates SMC contraction through the tubuloglomerular feedback and myogenic responses (see [62] for review). The former mechanism is mediated by increased glomerular filtration and NaCl delivery from the loop of Henle to the macula densa (MD), a cluster of epithelial cells located at the junction between the distal convoluted tubule and the end of the thick ascending limb and adjacent to the abluminal SMCs of the afferent arterioles. Increased NaCl uptake by MD cells results in secretion of ATP and adenosine which stimulate afferent arteriole SMC contraction via P2Y4/P2Y6 and A2 GPCRs, respectively[63-68]. The myogenic response is mediated by the activation of stretch-sensitive cation channels. Together these mechanisms stabilize renal blood flow to protect the sensitive glomerular capillaries from flow-induced trauma. Importantly, afferent arterioles express RhoA, ROCK I and II[68], and several studies have convincingly demonstrated that the Rho/Rho kinase pathway influences both of these feedback mechanisms in response to increased kidney perfusion[63-67]. The requirement of RhoA is likely due, at least in part, to its necessity for P2Y4/P2Y6 and A2 receptor-dependent contractility. Indeed, ATP (via P2Y4/Y6) and adenosine (via A2) stimulate RhoA activity in SMC and their pressor responses were prevented by pretreatment with the Rho-kinase inhibitor, Y-27632[68].

Interestingly, recent evidence indicates that RhoA may play an additional role in other cell types within the kidney to impact volume homeostasis. In particular, RhoA activity in tubular epithelial cells can regulate sodium reabsorption and excretion primarily by altering the density and location of epithelial sodium channels (ENaCs) and the sodium-hydrogen exchanger (NHE3)[69]. *In vitro* studies in cultured epithelial cells indicated that the Na^+ current through

ENaCs was significantly increased by expression of wildtype or constitutively active RhoA (G14V) and suppressed by expression of dominant negative RhoA (T19N). The changes in current correlated with alterations in the density of ENaCs at the PM[70] and mechanistic studies determined that RhoA signaling was essential for intracellular vesicle mediated transport of ENaCs to the apical cell surface[71, 72]. RhoA signaling also regulates the activity and subcellular localization of NHE3, a key regulator of sodium absorption in the proximal convoluted tubule. NHE3 associates with ezrin and cortical actin filaments at the plasma membrane and treatment with either the RhoA inhibitor, diarrheal toxin *toxin B*, or Y-27632 disrupted these interactions and promoted the internalization of NHE3 to sub-membrane compartments[73, 74]. Moreover, Nishiki *et al.* showed that spontaneously hypertensive rats exhibited elevated NHE3 activity and an exaggerated level of Na^+ reabsorption when compared to normotensive controls and that Na^+ reabsorption was normalized by treatment of the hypertensive animals with Y27632[75].

1.2.3 RhoA in the central and peripheral nervous system

The central nervous system (CNS) constantly assesses pressure levels in the vasculature and makes necessary signaling adjustments to prevent BP variability. The main mechanism by which the CNS monitors BP is through a rapid negative feedback loop termed the baroreceptor reflex. Baroreceptors are sensory neurons located primarily in the aortic arch and carotid sinuses that continuously respond to pressure-induced stretching of the vessels in which they reside. Impulses from baroreceptors are relayed via glossopharyngeal and vagus nerves to the nucleus tractus solitarii (NTS) in the brainstem[76], which in turn relays the signal to the rostral ventrolateral medulla[77] and increases or decreases parasympathetic and sympathetic stimulation to the heart and vessels accordingly. Interestingly, the CNS component of this feedback loop has

been shown to be dependent on RhoA/Rho-kinase signaling. Rho-kinase inhibitors microinjected directly into the NTS or infection of this structure with an adenovirus expressing a dominant-inhibitory form of Rho-kinase reduces sympathetic nerve activity, heart rate, and BP in normotensive rats and these effects are even more pronounced in spontaneously hypertensive rats[78, 79]. Moreover, infusing the ROCK inhibitor, Y27632, into the neural cistern attenuated the BP increase that resulted from AII infusion into the same area of the brainstem[80].

Recent studies indicate that the RhoA pathway may also regulate neurotransmitter release from perivascular nerves. Some studies in cells and invertebrate model systems indicate that inhibition of Rho/Rho kinase signaling in motor neurons antagonized the secretion of parasympathetic relaxation factors (including acetylcholine) and promoted the secretion of sympathetic contractile agonists (including dopamine)[81, 82]. Yamaguchi *et al.* found that $G\alpha_{12/13}$-mediated activation of RhoA/ROCK inhibited Ca^{2+} dependent exocytosis[81]. In support of these studies, an activating mutation in ArhGEF10, a RhoGEF highly expressed in the peripheral nervous system, was identified in patients who exhibited slowed nerve conduction velocities[83, 84]. Thus, it is possible that RhoA's ability to block neurotransmitter release in peripheral nerves could affect vascular tone and BP but such outcomes may limit the therapeutic efficacy of RhoA/ROCK inhibitors as future anti-hypertensive therapies.

1.2.4 RhoA in the myocardium

Several studies have shown that RhoA signaling has direct effects on cardiac function that increase cardiac output and BP. Transgenic mice that overexpressed either GDIα or dominant negative RhoA exhibited conduction defects and cardiomyocytes isolated from these mice exhibited decreased L-type Ca^{2+} channel currents that likely contributed to the decreased

contractility observed *in vivo*[85, 86]. Vlasblom *et al.* showed that treatment of neonatal ventricular cardiomyocytes with Y27632 reduced the expression and activity of the sarcoplasmic reticulum Ca^{2+} ATPase, SERCA2a, thereby limiting the amount available for Ca^{2+} -induced Ca^{2+} release in the next cardiac cycle[87]. In addition, RhoA-dependent pathways have been shown to be critical for phosphorylation and sensitization of cardiac troponin T complex to intracellular Ca^{2+} levels[88]. Moreover, while not initially thought to be a major mechanism for modulating cardiac contractility, it is becoming clear that cardiac MLC phosphorylation can enhance muscle contractility by increasing Ca^{2+} sensitivity [89]and that MLC phosphatase is a target for Rho kinase-dependent inhibition in the myocardium (like in SMC). Indeed, Lauriol *et al.* showed that cardiac-restricted deletion of RhoA led to decreased contractility and this effect was correlated with decreased MLC activity[90]. Other similarities between RhoA signaling in cardiomyocytes and SMC include the ability of RhoA-mediated signals to promote differentiation/maturation by promoting the expression of contractile genes[91].

1.3. Targeting the RhoA pathway for therapeutic blood pressure control

In agreement with the pre-clinical animal studies highlighted above, several lines of evidence strongly implicate RhoA signaling in the development of human HTN. First, increased Rho-kinase activity has been observed in some hypertensive patient populations, reviewed below. Rho kinase inhibitors have been successful in reducing systemic HTN in these cases, although current formulations exhibit relatively short-term effects[23, 92, 93]. Second, an autosomal dominant mutation in the E3 ligase, Cullin-3 (which targets RhoA), has been shown to cause high BP in patients with Gordon's Syndrome (pseudohypoaldosteronism type IIE). Importantly, the identical mutation in pre-clinical mouse models and led to decreased Cullin-3 activity,

reduced ubiquitin-mediated RhoA degradation in vascular SMCs, and increased BP[94, 95]. Third, many GWAS conducted over the past decade have identified common BP-associated genetic variations in coding and non-coding regions within or near genes linked to the RhoA signaling cascade. For example, in one study that used HTN as a dichotomous trait, two of the eight BP-associated loci were located in RhoA-related genes. One was within RhoBTB1, which functions with the aforementioned Cullin-3 complex to maintain low RhoA levels[94, 96], while the other was within rhotekin-2 (RTKN2), a RhoA effector with unknown function. Two separate GWAS identified a BP-associated locus within PLEKHA7 (Plekstrin Homology domain containing family A member 7)[97, 98] which interacts with the junctional proteins cingulin and paracingulin to regulate several Rho family GTPases, including RhoA in the heart and kidneys[99]. Importantly, PLEKHA7 was subsequently shown to be required for the development of salt-induced HTN in mice[100]. Moreover, a few variants in ROCK II have been associated with the regulation of BP. Of particular interest was the identification of a common nonsynonymous ROCK II variant 431N (versus 431T) that was associated with an increase in BP in twins[69, 101-104]. This result was supported by Liao *et al*., who showed that the 431N variant had increased kinase activity and was associated with enhanced arterial stiffening, a vascular property strongly associated with HTN[69, 101-104]. This group identified a second variation in the ROCK II 3'UTR (rs9789060) that was also associated with increased stiffening and went on to show it affected ROCK II expression by interfering with miR-1183-dependent degradation of ROCK II mRNA levels. It is important to note that a third study failed to find an association between rs9789060 and BP[101]. In another study on 586 normotensive and 607 hypertensive Caucasians, Rankinen *et al*. identified a minor allele locus within the ROCK II gene that lowered the risk of HTN by 85%. [103]. Finally, as discussed in further detail below, three separate GWAS for BP and cardiovascular disease

endpoints identified a novel BP associated locus within the Rho-specific GAP, GRAF3/ArhGAP42[15, 16, 105, 106]. Recent causality studies from our group demonstrated that GRAF3 is selectively expressed in SMC and is required for BP homeostasis in mice[36-38].

Collectively, these studies reveal that the RhoA signaling axis may provide tractable targets for the treatment of human HTN and related cardiovascular sequela. Indeed, some commonly used anti-hypertensives (i.e. ACE inhibitors, AII blockers, and statins) likely function by interfering with RhoA signaling, supporting the clinical utility of inhibiting this pathway[22, 107-109]. Nonetheless, despite the importance of the RhoA pathway in the pathogenesis of HTN and several other debilitating diseases including amyotrophic lateral sclerosis, mental retardation, hepatocellular, lung, and colorectal carcinomas[110-112], surprisingly few treatments are available to directly target this pathway. In fact, of the nearly 300,000 ongoing clinical trials, only a handful involve compounds that target RhoA signaling components (clinicaltrials.gov). This could be due to the fact that many members of the RhoA pathway (with exception of Rho kinase) have traditionally been regarded as "undruggable". However, as described below, significant advancements in high resolution crystal structures, structure-function analyses and drug development technology are beginning to overcome these challenges and provide hope for the development of new therapies to target this critical pathway.

1.3.1 Targeting Rho Kinase

The serine/threonine kinases, ROCK I and ROCK II, are the best studied RhoA effectors and have been implicated in a variety of diseases including HTN[23]. Since the development of kinase inhibitors has proven to be a very successful therapeutic approach, it is not surprising that ROCK has been an attractive target in the search for RhoA signaling inhibitors and that ROCK

inhibitors are the furthest along in regard to clinical testing. Because ROCK I and II share 60% identity overall, 90% identity within the kinase domain identity, and 100% identity within the ATP binding pocket[113] they share many substrates and promote many of the same downstream cell functions[93]. Although ROCK I and ROCK II expression can vary somewhat between tissues, both of these kinases are widely expressed[113, 114]. Differences in subcellular localization have been noted with ROCK I localizing more readily to microtubule-organizing centers[115] and catenin/E-cadherin containing complexes at the plasma membrane[116] and ROCK II to vimentin [117] and actin fibers[118 114, 119]. Isoform-specific inhibitors are being developed that could have differential effects depending upon the disease treated or end-point measured[23, 120-123] (clinicaltrials.gov).

While over 30 common downstream targets of ROCK have been identified (Adducin, Diaphanous [mDia], LIM kinase [LIMK], NHE1, MARCKS, NF-L, CRMP2, FAK, c-Jun N-terminal kinase [JNK], MLC, MLCK, MLCP, ezrin/radixin/moesin [ERM], rhophilin, rhotekin, citron kinase, and Tau)[23, 93, 119, 124], the most pertinent in regard to SM contraction and BP regulation is the myosin binding subunit (MYPT-1) of the MLCP complex. ROCK-dependent phosphorylation of MYPT-1 at T696, T853, and S854 inhibits MLCP activity resulting in increased MLC phosphorylation and hence increased contraction[114, 125]. ROCK has also been shown to directly phosphorylate MLC at S19.

As reviewed more thoroughly elsewhere[23, 93], over 170 ROCK inhibitors are in various stages of development (**Table 1.1**). Given the involvement of actin-based processes in many pathologies (adhesion, migration, cell division, force generation, etc.), ROCK inhibitors are being studied as treatments for a wide variety of disease states including central nervous system disorders (subarachnoid hemorrhage and vasospasm, cerebral stroke, spinal cord injury),

neurodegenerative diseases (Alzheimer's disease, Parkinson's, Huntington disease, and amyotrophic lateral sclerosis [ALS]), cardiovascular disease (systemic HTN, pulmonary arterial HTN [PAH], spastic and stable angina, atherosclerosis, Raynaud syndrome), asthma, glaucoma, autoimmune diseases, cancer, erectile dysfunction, and kidney disease. Although the results of these studies and clinical trials provide important information on ROCK and its role in disease progression, we will focus our discussion on the major ROCK inhibitors that have been most commonly used to treat vascular diseases.

Fasudil (also known as HA-1077) was the first ROCK inhibitor described and was also the first to be tested clinically. This isoquinoline-based drug is classified as a class I ROCK inhibitor because it reversibly competes with ATP binding to the ROCK kinase domain. Fasudil inhibits both isoforms of ROCK with an IC_{50} of 1μM while hydroxyfasudil, the major active metabolite, is slightly more potent with an IC_{50} of ~0.7μM[126]. Like most other ROCK inhibitors of this class fasudil also inhibits other members of the broad AGC kinase family including PKA and PKC, albeit with less potency (IC_{50} of 5μM and 37μM, respectively)[126, 127]. Fasudil was shown to reduce BP by attenuating the Rho-mediated inhibition of MLCP in SMC[126], by increasing endothelial nitric oxide synthase expression[128], and by reducing circulating ACE and AII levels[129]. Fasudil also inhibited pulmonary artery SMC proliferation, a process important for vessel stiffening, by a mechanism that likely involved downstream inhibition of c-Jun N-terminal kinase (JNK) and ERK-dependent activation of c-jun and c-fos expression[130].

Fasudil hydrochloride hydrate under the trade name Eril® was approved in Japan in 1995 to treat vasospasm-induced cerebral ischemia that can occur following surgery for subarachnoid hemorrhage. In 2002, Masumoto *et al*. showed that intracoronary fasudil was effective as an acute treatment of vasospastic angina[131], and additional clinical trials showed that long-term

treatment with oral fasudil reduced stable effort-induced angina and improved exercise tolerance with no major adverse effects[132, 133]. In a rat model, moderate doses of fasudil decreased PAH while higher doses also decreased mean systemic arterial pressure[134]. By 2011, intravenous and inhaled fasudil were approved to treat PAH[135, 136], and a new extended release formulation of fasudil, AT-877ER, was shown to reduce PAH in patients after three months of use[137]. Fasudil also decreased forearm vascular resistance more dramatically in hypertensive patients than in normotensive controls[21]. Fasudil has been shown to have beneficial effects on kidney function in diabetic rats[138], suggesting that it might be useful for treating diabetic patients who are frequently hypertensive and have kidney disease. Ongoing phase III clinical trials are also assessing whether fasudil is an effective treatment for Reynaud's syndrome and carotid stenosis (clinicaltrials.gov). Fasudil has been approved in China, but not in the United States or Europe.

Several fasudil derivatives that are more potent and specific inhibitors of ROCK have been developed and are being used to treat glaucoma and ocular HTN. Ripasudil (Glanatec®), approved in Japan in 2014, affects the trabecular meshwork in the eye and reduces intraocular pressure by facilitating the outflow of aqueous humor through Schlemm's canal. The most common side effect of ripasudil treatment is conjunctival hyperemia, which subsides over time or with discontinued use[139]. Netarsudil (Rhopressa ®) has similar effects and indications, and in 2017, became the first ROCK inhibitor approved by the United States[140, 141]. Netarsudil also decreased the amount of aqueous humour produced, a feature attributed to additional effects of netarsudil as a norepinephrine transport inhibitor[140, 141].

The classic ROCK inhibitor, Y-27632, is a pyridine-based class I inhibitor that has been tested in a number of animal and human disease models including HTN. In a landmark study, Uehata *et al*. demonstrated that Y-27632 decreased BP in spontaneously hypertensive rats

(SHR), DOCA-salt treated rats, and rats made hypertensive by clipping of the renal artery[142]. Although mesenteric and cerebral arteries in SHR or DOCA/salt treated rats were more responsive to Y-27632 than those in normotensive rats[143, 144], Y-27632 likely affected HTN by multiple mechanisms. For example, Y-27632 also reversed the decrease in renal sodium excretion observed in the SHR model[75], most likely by affecting the activity and location of Na+/H+ exchanger, NHE3[73, 74]. In addition, local infusion of Y-27632 into the nucleus tractus solitarius of the brainstem caused a reduction in BP, heart rate, and renal sympathetic nerve activity, and the magnitude of these effects was greater in the SHR model[78, 79]. Subsequent studies by the same group demonstrated that fasudil had similar effects[78, 79]. Although Y-27632's poor potency and kinase selectivity limit its use clinically, Y-27632 derivatives with better pharmacologic properties are being developed and tested.

SAR407899 is a promising relatively new isoquinoline-based class I ROCK inhibitor that is significantly more potent than older generation drugs (IC_{50} between 122 and 280 nM)[145]. *In vitro* studies demonstrated that SAR407899 inhibited myosin phosphatase phosphorylation, stress fiber formation, cell proliferation, and monocyte chemotaxis[145]. SAR407899 dose-dependently lowered BP in SHR, stroke-prone SHR, L-N^G-Nitroarginine methyl ester (L-NAME), and DOCA-salt rat models, and its effects in some models was superior to ACE inhibitors and calcium channel blockers[145]. SAR407899 inhibited pressor responses to PE, AII, and vasopressin in rats more strongly than Fasudil or Y-27632, and it inhibited ET1-induced vasoconstriction of renal arteries isolated from diabetic rats more strongly than Y-27632[146]. In spite of its efficient antihypertensive effects and the fact that long term treatment of rats with SAR407899 was well-tolerated, development of SAR407899 as an anti-hypertensive has been discontinued[23]. SAR407899 is still being tested in clinical trials as a treatment for kidney disease

and microvascular coronary artery disease and it may prove useful for treatment of erectile dysfunction in diabetic and hypertensive patients where the eNO system is impaired[147].

Although ROCK inhibition has been shown to reduce BP and vascular resistance in many models, there are concerns about the suitability of ROCK inhibitors as viable long-term treatments for systemic HTN. Although most ROCK inhibitors seem to be fairly well-tolerated, the ubiquitous nature of ROCK I and ROCK II expression coupled with the relative lack of specificity of most ROCK inhibitors (especially the class I drugs) makes potentially unknown side-effects a significant drawback. While local delivery strategies can sometimes mitigate this concern (i.e. to the eye or specific vascular beds)[93, 148], systemic hypotension can be a serious problem for patients being treated in this manner. Another potential problem is that the systemic BP lowering effects of ROCK inhibitors frequently decrease after 7-10 days of chronic treatment[93]. Moreover, ROCK inhibitors did not affect systolic BP in some long-term studies of salt-sensitive hypertensive Dahl rats[69, 75]. The precise causes of this tolerance or inactivity are unknown, but likely involve compensation by the many feedback pathways that regulate BP. Finally many ROCK inhibitors have short half-lives, which is not ideal for the treatment of a chronic disease like HTN[149].

1.3.2 Targeting RhoA directly

Numerous failed attempts to identify small molecule inhibitors of H-Ras have led to the concept that small GTPases per se do not make good drug targets due to their globular structure and lack of surface moieties required for high affinity binding of small molecules[110, 150, 151]. However, such direct targeting may be the most effective means to reduce signal output given that many disease-associated mutations of small GTPases enable GEF-independent activation[152,

[153]. Moreover, in the case of HTN, direct targeting could provide an added benefit over ROCK inhibitors, as such an approach would block additional downstream pathways implicated in SMC contractility (i.e. mDia1 and mDia2, MRTF, *etc.*).

1.3.2a GTP-binding inhibitors

Although direct targeting of the GTP binding site of RhoA (or related small GTPases) is challenging due to its high affinity for GTP (in the pico to nano-molar range) and the high concentration of GTP in cells (~0.5 mM), some studies support the validity of this approach. Indeed, using an *in silico* virtual docking approach followed by surface-plasmon resonance validation of synthesized chemicals, Deng *et al.* identified lead compounds that inhibited Rho-GTP binding in a dose-dependent fashion with IC_{50} values ranging from 1.24–2.05 μM[154]. After further structural modifications to increase water solubility, one compound ((*E*)-3-(3-(ethyl(quinolin-2-yl)amino)phenyl)acrylic acid) was shown to both attenuate PE-induced contraction in thoracic aorta rings *ex vivo* and to reduce cerebral vasospasm in a subarachnoid hemorrhage model in rats[155]. While the *in vivo* efficacy was similar to that of fasudil, this second generation inhibitor still exhibited relatively low potency (IC_{50} 71 μM in the contractile assay). Future studies will be necessary to determine whether these or related compounds exhibit specificity for RhoA versus other Rho family GTPases and other GTP-binding proteins.

In support of the possibility of identifying GTP binding inhibitors that can specifically target particular Rho-related family members, simultaneous multiplex screening for small molecules in the 200,000 Molecular Libraries Screening Center Network library has already identified a CDC42 specific inhibitor as well as a broad Ras family inhibitor. These inhibitors prevented GTP binding in a dose-dependent fashion (as noncompetitive allosteric inhibitors) and

are active in cell based assays[156-158]. The subsequent application of this technology to screen the Prestwick Chemical Library of off patent and FDA approved drugs for inhibitors of eight Ras-related GTPases (but not RhoA, B, or C) led to the identification of R-enantiomers of naproxen and ketorolac (approved NSAIDs) as GTP binding inhibitors of Rac1 and Cdc42[159]. Although not biochemically confirmed, *in silico* docking analyses predicted that these drugs bind to an allosteric site near the GTP binding site that alters Mg^{2+} ion coordination and results in stabilizing the GTPase in its GDP-bound form. To our knowledge, this approach has not been used successfully to identify RhoA specific inhibitors, but these results provide strong proof-of-concept for this approach.

1.3.2b Selective non-covalent RhoA modifiers

The potential for identifying allosteric modifiers to inhibit RhoA has been demonstrated by the fact that a number of bacterial toxins are highly potent (though non-selective) inhibitors of Rho family members. For example, *Histophilus somni* and *Vibrio parahemolyticus* produce toxins that inhibit Rho proteins by promoting the covalent attachment of an AMP molecule to tyrosine 34, while toxin B produced by *Clostridium difficile* induces glucosylation of nearby threonine 37. These residues lie within the regulatory switch-I domain of Rho family members and addition of these bulky modifications in this domain inactivate the Rho GTPases by multiple mechanisms that include inhibition of GTPase cycling (by blocking GEF and GAP association), inhibition of cytosol-membrane cycling (by blocking Rho GDI interactions) and inhibition of Rho effector coupling[160, 161]. Similarly, *Clostridium botulinum* exoenzyme C3 transferase toxin inhibits RhoA, RhoB, and RhoC *in vitro* and *in vivo* by promoting ADP-ribosylation of asparagine 41 which blocks GEF binding[110, 162]. Such pathogenic agents have been useful as

pharmacological tools to completely block Rho-dependent signaling pathways in cells and in pre-clinical animal models. However, their potential as therapeutic agents is limited because of their difficult delivery, their non-specific actions, and their sometimes covalent and irreversible effects[110, 163]. Nonetheless, the future exploitation of derivatives or mimetics of such enzymes could lead to the development of new inhibitors with tremendous clinical utility.

Finally, Rho family inactivation can be achieved by blocking plasma membrane targeting. For example, Rho GTPases are isoprenylated on carboxy-terminal Cysteine residues (within a so-called CAAX box) and this modification is important for membrane targeting and activation as evidenced by the fact that proteolytic cleavage of this site by *Yersinia* spp.-derived toxins effectively block Rho, Rac and CdC42 activation[161]. Likewise, as RhoA is geranylgeranylated at this site, geranylgeranyl-transferase inhibitors and HMG-CoA-reductase inhibitors (which block both cholesterol and isoprenyl biogenesis) block RhoA membrane association and activation. In fact the clinical utility of this approach is highlighted by the fact that HMG-CoA reductase inhibitors such as simvastatin and atorvastatin, used to treat high cholesterol, also have anti-hypertensive properties[109] and their BP lowering effects have been attributed to their ability to block RhoA signaling[108, 109, 164]. However, the BP lowering effects of statins are modest (2 mmHg decrease systolic BP) and these isoprenoid pathway inhibitors display poor selectivity for individual Rho GTPases. Thus, further advancements in drug development are needed to realize the full potential of this approach.

1.3.3 RhoA GEFs and blood pressure control

Another potential strategy for inhibiting RhoA signaling is inhibition of the GEFs that control RhoA activity. Over 24 Rho-specific GEFs have been identified, and to date, at least 5 of

these have been implicated in regulating SMC differentiation and/or contractility. This list includes 3 members of the RGS-GEF subfamily (P115-RhoGEF, PDZ-RhoGEF, LARG), p63RhoGEF and lymphoid blast crisis (Lbc)[20, 165-168]. Each of these RhoGEFs contains a Dbl homology (DH) domain (also known as the RhoGEF domain) followed by a pleckstrin homology (PH) domain (**Table 1.2**). The DH domain serves as both the catalytic site and the major binding interface for RhoA, while the PH domain facilitates membrane binding and cooperates with DH domains to fully activate RhoA. Other common domains include a regulator of G-protein signaling (RGS) domain that binds heterotrimeric G proteins, or the density 95, disk large, zona occludes-1 (PDZ) domain that binds to specific transmembrane receptors (including the Lysophosphatidic Acid (LPA) receptor among others[169]. Importantly, these 5 GEFs are all highly expressed in conductance and resistance arteries of rats, mice, and humans[20, 165-168] (Genotype-Tissue Expression [GTEx] Portal, accessed on 07/12/2018).

The regulator of G-protein signaling (RGS) family of Rho GEFs (LARG, p115RhoGEF, and PDZRhoGEF)[170] has received a lot of attention in the BP field because these proteins are activated by $G\alpha_{12}$ and $G\alpha_{13}$- coupled receptors which transduce signals mediated by major contractile agonists that include AII, PE, ET1, and thromboxane A2[171]. P115RhoGEF (p115) is the critical GEF that mediates AII-dependent RhoA activity in SMC and small arterioles, and importantly, Guilluy *et al.* showed that SM-specific deletion of p115 rendered mice resistant to AII-dependent HTN[35]. However, AII–dependent activation of RhoA in SMC has also been linked to activation of LARG[172], PDZ-RhoGEF[165, 167], and p63RhoGAP[173] and inhibition of p190RhoGAP[174] (see below), suggesting significant overlap between these pathways. For example, Ying *et al.* showed that Ca^{2+}/PYK2-dependent activation of PDZ-RhoGEF was necessary for maximal A-II induced RhoA activation in SMC[167]. Alternatively, p63-RhoGEF,

which is highly expressed in arterial SM, was shown to be important for the early phase of AII-dependent vessel contractility[173] and for maximal pressor response to other vasoconstrictors such as PE and ET1 that act through $G\alpha_{q/11}$[175]. Interestingly, p115 mutant mice exhibited normal pressor responses to ET1 and PE, but did not respond fully to AII and had a partial reduction in DOCA/salt- induced HTN. On the other hand, LARG knockout mice were fully resistant to salt-induced HTN[20], which is consistent with subsequent findings that LARG regulates RhoA activity and SMC contractility in response to mechanical forces—which are known to be applied to small vessels in this volume overload model[176]. While not yet confirmed in pre-clinical animal models, the non-RGS Rho GEF termed lymphoid blast crisis (Lbc) is necessary for serotonin-dependent activation of RhoA and contractility in vascular SMC[177]. Thus, specific vasoconstrictors can lead to activation of distinct but overlapping sets of RhoGEFs to enable the fine-tuning of vessel tone and BP homeostasis.

With respect to pharmacological treatments, these findings indicate that targeting a critical SM GEF might provide greater therapeutic benefit than targeting RhoA or Rho kinase, because it could lead to a more modest (and cell type restricted) reduction of RhoA activity and result in fewer side effects. Moreover, the use of validated inhibitors could provide 'personalized' approaches that would better target the underlying pathophysiology (*i.e.* to limit HTN due to volume overload or elevated sympathetic activity). On the other hand, known functional redundancies also suggest that it may be necessary to target multiple GEFs to achieve therapeutic efficacy.

1.3.3a Targeting RhoGEFs

GEF protein-dependent nucleotide exchange involves a multi-step reaction. Upon binding to the GDP-bound form of the GTPase, GEFs facilitate release of GDP resulting in the formation of a nucleotide-free GTPase–GEF transition complex. The reaction then terminates with the binding of GTP (which is in a 10:1 molar excess in the cell) and dissociation of the GEF from the now active GTPase. The implication of this multi-step process for drug discovery is that these transition-binding intermediates tend to have the characteristics of druggable hotspots (*i.e*. exposed hydrophobic surfaces, unpaired polar groups, deeply curved surfaces, *etc*.) while the unbound, inactive proteins do not[178]. Thus, it may be possible to identify small molecules that specifically target the GEF-GTPase interface (*i.e.* inhibit GEF binding or stabilize the GEF-GTPase transition state) or directly interfere with GEF activity.

1.3.3b Targeting the RhoA-GEF interface

Facilitated by high resolution crystal structures and sophisticated *in silico* screening, some recent drug discovery approaches support the possibility of targeting the RhoA-GEF interface. For example, Shang *et al*. virtually screened 4 million compounds for their ability to pack into the Rho-GEF binding surface groove of RhoA. One of the drugs identified (termed Rhosin) contains two aromatic rings tethered by a linker which wraps around RhoA AA58 (a critical Tryptophan that is essential for GEF binding) and prevents RhoA from interacting with LARG, DBL, LBC, p115RhoGEF and PDZ RhoGEF without interfering with RhoGAP, RhoGDI, or RhoA effector binding[151]. Rhosin reversibly inhibited serum-induced RhoA, RhoB and RhoC activity, but did not inhibit other Rho GTPases (Rac1 or Ccd42)[151]. Rhosin also significantly and dose-dependently inhibited several RhoA-dependent functions in cells

including MLC and PAK phosphorylation and stress fiber and focal adhesion formation[151]. Rhosin suppressed invasion and mammary sphere formation in breast cancer cells[151], and also mitigated the acquisition of drug resistance in cancer stem cells[179]. The ability of Rhosin to impact either BP homeostasis (or cancer growth) has not yet been tested in animal models. Since Rhosin exhibits a relatively low binding affinity, (K_d ~0.4 μM for RhoA binding *in vitro*) the identification of derivatives with better pharmacologic properties will be needed before Rhosin compounds can be tested clinically[151]. Moreover, since Rhosin binds to RhoA and prevents all GEFs from binding to this site, this drug is likely to have more off-target effects than one that could interfere with specific GEF complexes.

The first proof-of-concept for targeting a specific GEF-GTPase interface was a series of elegant studies that unveiled the mechanism by which the fungal toxin, Brefeldin A (BFA), inhibited Arf1-dependent trafficking of proteins from the endoplasmic reticulum to the Golgi. Biochemical studies revealed that BFA selectively blocked the ability of the Arf1GEF, Sec7, from catalyzing GDP release. Importantly co-crystallization studies revealed that BFA binds to a hydrophobic pocket that does not exist in $Arf1^{GDP}$ but is created upon Sec7 binding. Since this energetically unfavorable hydrophobic pocket drives the conformational changes necessary for nucleotide dissociation, BFA binding effectively locks the complex in a GDP bound conformation[180-182]. In spite of high sequence homology among the Arf1GEFs, only the Sec7 complex is targeted by BFA, suggesting that 'interfacial' small molecule inhibitors could be identified that specifically target RhoA-GEF interactions.

These studies have inspired a new line of investigation that seeks to exploit novel pockets at small GTPase-GEF interfaces. The most productive approach so far used NMR-based fragment screening combined with high resolution structure/functional analyses to identify small

molecule inhibitors of kRas and its GEF, Sos1. These screens identified compounds that either block Sos1 binding to kRAS or that bind to a site adjacent to the functionally important switch I/II regions of KRas and (like BFA) block SOS1-dependent nucleotide exchange. A similar approach was used to identify a ligand that binds to the cavity adjacent to the switch II region of RhoA and inhibits the interaction between $RhoA^{GDP}$ and the LARG DH domain[183]. Small molecules have also been identified that inhibit TrioGEF's interaction with Rac1 and RhoG and Cdc42's interaction with the GEF, intersectin-1[184]. Clearly much work needs to be done to realize the potential for such ligands. The inhibitors identified exhibit very low potency (in the high micromolar range) and their ability to block additional GEFs has not yet been evaluated. In fact, *in silico* evaluation of aforementioned SOS1 inhibitor suggests that the hydrogen bonds formed by this ligand in the Ras-SOS1 complex would likely be conserved in the complex of Ras and another cognate GEF, RasGRF1[183]. While challenging, further medicinal chemistry and molecular dynamics approaches should facilitate our ability to target RhoA's interaction with specific GEFs.

1.3.3c Targeting GEF activity

Another viable approach is to directly target the activity of specific RhoGEFs before they interact with the small GTPases. Using a high-throughput screen, Shang *et al.* identified a compound, Y16, that binds LARG at the junction between the DH and PH domains with a K_d of ~80nM[185]. Y16 prevented LARG from interacting with RhoA *in vitro* and reversibly attenuated serum-induced RhoA activity in NIH 3T3 cells and mammary sphere formation MCF7 cells with little to no toxicity[185]. A separate fluorescent ligand-based screen of ten thousand compounds yielded five additional selective inhibitors of LARG-dependent RhoA GTPase activity, though

their mechanisms of action have not yet been determined[186]. Again, to date, many of these lead compounds exhibit low potency, but theoretically, combination therapy strategies which pair Y16 with some of these compounds and/or their derivatives may lead to a highly efficacious approach to limit LARG-mediated signaling.

Finally, since many GEFs are regulated by protein-protein interactions and post-translational modifications, it may be possible to identify drugs that block these mechanisms. In an excellent example of this approach, cAMP is a potent activator of the Rap1 GEFs, Exchange Proteins directly Activated by cAMP (EPAC1 and 2) and high throughput screens to identify small molecules that displace cAMP binding led to the discovery of several inhibitors or partial agonists for these GEFs[187-189]. Such studies support the concept that identification of small molecules that prevent physiological activation of specific GEFs could prove therapeutically useful. Along these lines, several studies have shown that RGSRhoGEFs are regulated by phosphorylation. Guilluy *et al.* demonstrated that AII-dependent activation of p115GEF was mediated by phosphorylation of the PH domain (Tyr738) by Janus tyrosine kinase[22]. Importantly, phosphorylation mimetic and deficient variants of Tyr738 elevated and reduced p115's GEF activity, respectively. PDZRhoGEF and LARG are also activated by tyrosine phosphorylation. Focal adhesion kinase (FAK) and its related family member, proline-rich tyrosine kinase 2 (PYK2) phosphorylate and activate PDZRhoGEF[167, 190], while FAK, Tec and the serine-threonine kinase, p90 ribosomal kinase-2 each phosphorylate and activate LARG[167, 170, 190, 191]. Drugs that specifically inhibit these phosphorylation events could prove to be useful RhoA signaling inhibitors.

1.3.4 RhoA GAPs and blood pressure control

Although GEFs have classically been considered to be the major regulators of RhoGTPase activity, increasing evidence suggest the GAPs are also critically important. As their name implies, GAPs enhance the intrinsic GTPase activity of the Rho GTPases by several orders of magnitude thus decreasing the length of time that GTPases are in the active form[192-194]. The 66 RhoGAPs in the human genome comprise a broad and diverse family that can be further subdivided based upon the presence of a variety of functional domains[195] (**Table 1.3**). By mediating interactions with different membrane and protein components these domains are critical for the selective function of the GAP proteins and the dynamic inhibition of small GTPase signaling. Several Rho-selective GAPs including p190ARhoGAP, ArhGAP1, Myr5, GRAF1, and GRAF3 have been shown to inhibit RhoA activity in cultured vascular SMC and could be potential targets for therapeutic interventions that affect vascular function and BP[36, 196]. However, GRAF3 is the only RhoGAP that has been directly linked to the regulation of SM contractility and BP homeostasis[15, 16, 36, 105, 106].

1.3.4a GRAF3 and Hypertension

We originally identified the founding member of the GRAF (GTPase Regulator Associated with FAK) family by screening an embryonic λgt11 expression library for proteins that interacted with the carboxyl-terminal domain of FAK[197-199]. This family which is now known to comprise 3 members, GRAF1 (ArhGAP26), GRAF2 (ArhGAP10) and GRAF3 (ArhGAP42) contain an N-terminal BAR (Bin/amphiphysin/Rvs) domain, a phosphatidylserine (PS)-binding PH domain, a central Rho-GAP domain, a serine/proline rich domain, and a C-terminal FAK-binding SH3 domain[197]. GRAF1 is expressed predominantly in the brain and

striated muscle (cardiac and skeletal), and our studies in GRAF1-depleted *Xenopus* and mice revealed that GRAF1-dependent inhibition of RhoA activity promoted mammalian muscle growth by facilitating myoblast fusion and injury repair[199-202]. GRAF2 is more ubiquitously expressed[203] and could partially compensate for the loss of GRAF1 during myotube formation, supporting at least some functional redundancy within this family[201]. Evolutionarily, GRAF3 is the youngest family member and is the most recently annotated. Importantly, we found that GRAF3 was highly and selectively expressed in SMC with particularly high expression in resistance vessels[36] suggesting that its expression levels might be a major determinant of RhoA activity, SM contraction, and vessel tone.

Over the last several years, studies from our laboratory confirmed that GRAF3 is a SMC-selective Rho GAP protein that imparts tight control of BP homeostasis by modulating vascular resistance. Specifically, we showed that mice with gene-trap-mediated reductions in GRAF3 levels exhibited significant basal HTN and elevated pressor responses to AII, ET1, PE, and DOCA salt[36]. Notably, the hypertensive phenotype in this model was fully reversed by treatment with ROCK inhibitors or by Cre-mediated re-expression of GRAF3 in SMC, strongly supporting the contention that GRAF3 control of SMC RhoA activity is necessary for homeostatic control of basal and pressor-induced BP. Mechanistically, our data supported a model wherein GRAF3 controls BP homeostasis by limiting RhoA-dependent MLC phosphorylation and blunting acute Ca^{2+}-mediated SMC contractility in resistance vessels. Moreover, depletion of endogenous GRAF3 from vascular SMC enhanced MRTF-A nuclear accumulation, and stimulated expression of contractile proteins including SM α-actin, SM-myosin heavy chain, calponin and SM-22, indicating that changing the levels of GRAF3 would likely have a long-lasting impact on vessel tone[37]. Interestingly, GRAF3 mRNA was significantly upregulated in SMC cultures

subjected to cyclic stretch and in isolated portal vein segments subjected to static stretch and we showed that these effects were mediated via the RhoA/MRTF/SRF pathway[37]. Since similar physical forces are known to be increased in the vessel wall under hypertensive conditions[55], we postulated that GRAF3 might serve as a transcriptionally mediated negative feedback loop of the RhoA signaling axis. In support of this possibility, arterial GRAF3 mRNA levels were significantly increased in mice made hypertensive by L-NAME or DOCA-salt regimens[36]. In fact, taking advantage of an elegant mouse model (of aldosteronism) developed by the Smithies lab in which plasma volumes of mice range from ~50% below normal to ~50% above normal[204], we showed that GRAF3 expression increased in parallel to and was strongly correlated with plasma volume (r^2=0.94)[37]. Taken together, these findings suggested that GRAF3 serves as a mechanical strain-sensitive rheostat that acts to prevent excessive feed-forward activation of the RhoA signaling axis in order to control SMC tone and BP.

Of clinical importance, three separate GWAS studies identified a blood-pressure associated allele within the GRAF3 locus. Notably, our recently published follow-up studies revealed that GRAF3 mRNA levels were approximately 3-fold higher in arteries from individuals homozygous for the protective allele and our human genetic data from well-characterized untreated patients confirmed that the protective allele was associated with a 5mm Hg decrease in BP[15, 16, 38, 106] (Appendix A and Appendix B). We went on to identify a novel mechanism for the BP-associated locus which mapped to the first intron of the GRAF3 gene[38]. Our studies revealed that rs604723 is the causative SNP at this locus and that the minor T allele variation increases *GRAF3* expression by promoting SRF binding to a SMC-selective intronic regulatory element. Our demonstration that the minor *GRAF3* allele is more highly expressed in human aortic SMCs (HuAoSMCs), when coupled with similar data from human artery samples,

strongly supports our hypothesis that this variation reduces BP by inhibiting RhoA-dependent constriction of resistance vessels. Our data add to a growing body of evidence that common noncoding variants alter cardiovascular risk by altering transcription factor binding and gene expression and support previous studies implicating RhoA signaling in the regulation of BP homeostasis in mice. Moreover, when coupled with the remarkable SMC-selective expression pattern of GRAF3, these studies provide strong evidence for GRAF3 as a novel therapeutic target for HTN.

1.3.4b Druggability of RhoGAPs

To date, targeting GAPs for therapeutic advances has been largely overlooked. This is due, in part, because GAPs have little to no effect on promoting GTP hydrolysis of oncogenic Ras and Rho mutants, and therefore would not be good targets for cancer treatment[205]. GAPs have also been considered less attractive targets for anti-hypertensive therapies because the drug would need to enhance GAP activity and it is traditionally thought to be more difficult to develop small molecule activators than small molecule inhibitors. Nonetheless, due to the multi-domain nature and the varied physiological regulation of RhoGAP proteins, there are several possibilities for allosteric modulation of GAP activity. Indeed, the activities of several RhoGAPs including the GRAFs, OPHN1, β-chimerin, DLC1, and p50 Rho GAP are regulated by intramolecular auto-inhibition. For GRAF1 and similarly structured Oligophrenin and ASAP1, the BAR and PH domains physically associate with the GAP domain to sterically inhibit its function[206, 207]. We and others have shown that this mechanism also controls the activity of GRAF3 (see Chapter 2;[208]) (**Figure 1.2**). By defining the structural interactions that control GRAF3 activity, these

results could lead to the development of GRAF3 activators that target the BAR-PH/GAP interface that should be useful for reducing arterial tone.

1.4 Conclusions

In conclusion, the search for new approaches to control high BP remains dramatically important for reducing global health burden, and targeting RhoA-mediated SM contractility is a promising avenue (**Figure 1.3**). Indeed, strong evidence from pre-clinical animal models indicate that modulating the activity of SM Rho-GEFs, Rho-GAPs, or ROCK has a major impact on systemic BP homeostasis. While new advances in drug development have led to potent and specific ROCK inhibitors that can be safely used in patients, whether any of these compounds exhibit the necessary selectivity and pharmacological profiles required for BP management in patients requires further study. Although efforts to target GEF and GAPs or the RhoA binding interface for these enzymes are lagging behind, recent advances in drug discovery indicate that it will likely soon be possible to engineer clinically-relevant small molecule regulators of these enzymes that could be effective anti-hypertensive therapies. To ensure success in this regard, we will need to continue to gain a better understanding of the mechanisms that regulate these enzymes. Moreover, based on the fact that BP is a highly variable trait among individuals, a better understanding of the genetic mechanisms regulating this disease is critical for a more personalized treatment plan for patients. Given that genetic variations in both upstream activators and downstream mediators of RhoA have been linked to BP regulation, screening for such variants could potentially be used to tailor more effective individualized treatment regimens.

1.5 Objectives

Due to the smooth-muscle specificity of GRAF3 and the regulation of RhoA signaling that GRAF3 exhibits *in vitro* and *in vivo*, the first major objective of this dissertation was to assess the therapeutic potential of GRAF3 activation/overexpression to reduce basal blood pressure or combat HTN. The second objective of this work was to identify the biochemical mechanisms that regulate GRAF3 in SMCs. Expanding our understanding of how GRAF3 functions endogenously is pertinent for the screening and design of small molecule activators. More recently, our lab has discovered that in humans, GRAF2 is more highly expressed in SMCs than GRAF3 is. The third and fourth objectives of this dissertation, therefore, respectively, were to better characterize the role of GRAF2 in BP homeostasis and to evaluate the degree of redundancy (if any) between GRAF2 and GRAF3.

1.6 Figures

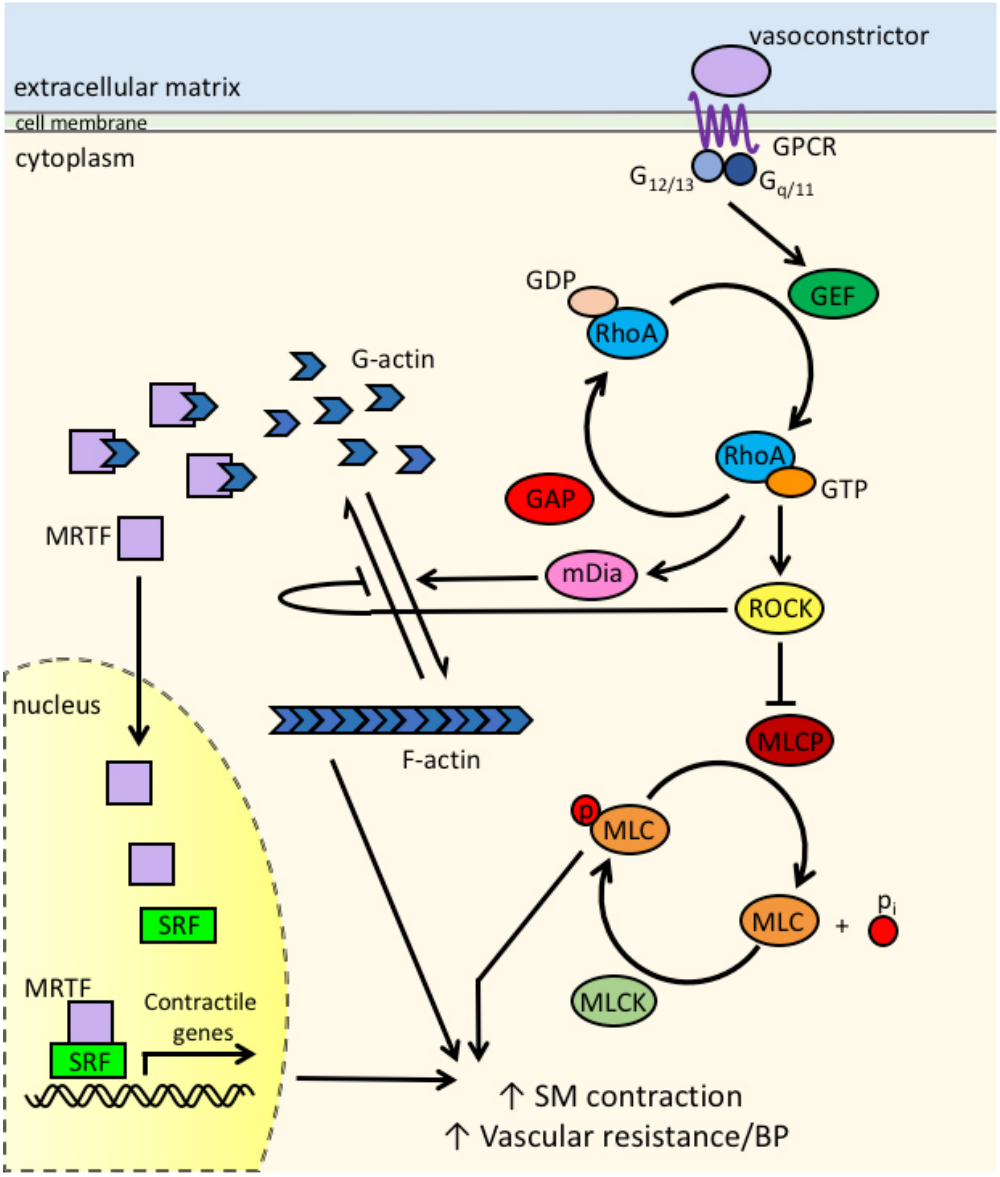


Figure 1.1 RhoA pathway in smooth muscle cells. In addition to Ca^{2+}-dependent activation of MLCK (not shown here), vasoconstrictors such as AII, PE, and ET-1 stimulate G-protein signaling in SMC via GPCRs. G-proteins influence the activity of RhoA by activating GEFs, which facilitate the dissociation of GDP from RhoA, allowing GTP to bind. This activation is balanced by GAPs, which increase the efficiency of GTP hydrolysis. When RhoA is in its active, GTP-bound form, it's able to interact with any of its downstream effectors, such as ROCK and mDia. ROCK inhibits MLCP, allowing for prolonged phosphorylation of MLC and enhanced SMC contraction, increased vascular resistance, and increased blood pressure. Simultaneously, mDia and ROCK promote the polymerization of F-actin from G-actin, releasing G-actin bound MRTF. Free MRTF translocates to the nucleus where it induces SRF-dependent transcription of contractile genes. The concurrency of these latter events further enhance SMC contraction and vascular resistance.

Drug	Disease	Phase	Status	ClinicalTrials.gov ID
Fasudil	Cerebral vasospasm, brain ischemia, stable angina, pulmonary HTN	NA	**Approved in Japan and China**	NA
	Raynaud/Scleroderma	3	completed	NCT00498615
	Carotid Stenosis	2	terminated	NCT00670202
	Atherosclerosis, Hypercholesterolemia	2	completed	NCT00120718
	Amyotrophic Lateral Sclerosis	2	uknown	NCT01935518
	Cardiovascular Disease	2	recruiting	NCT03404843
Ripasudil	Glaucoma and ocular HTN	NA	**Approved in Japan**	NA
	Fuchs' Endothelial Dystrophy	4	recruiting	NCT03249337
AR-12286	Chronic Angle-closure Glaucoma	2	unknown	NCT02152774
	Advanced Glaucoma	2	unknown	NCT02173223
	Glaucoma	2	unknown	NCT02174991
	Exfoliation Syndrome, Ocular HTN, Open Angle Glaucoma	2	completed	NCT01936389
AR-13324	Open-angle glaucoma, Ocular HTN	NA	**FDA approved**	NA
SAR-407899	Erectile dysfunction	2	completed	NCT00914277
	Microvascular Coronary Artery Disease	2	recruiting	NCT03236311
	Chronic Kidney Disease	1	completed	NCT01485900
KD-025	Psoriasis Vulgaris	3	completed	NCT02317627
	Psoriasis	2	recruiting	NCT02852967
	Graft vs. Host Disease	2	recruiting	NCT02841995
	Idiopathic Pulmonary Fibrosis	2	recruiting	NCT02688647
	Fibrotic Disease	1	recruiting	NCT03530995

Table 1.1 ROCK inhibitors currently in clinical trials. ROCK inhibitor compounds and presumptive applications are outlined here, along with the phase of the study, the status of the study, and the registered clinical trials identifier number. NA, not applicable

GEF Protein	Length (AA)	Domain			
		PDZ	RH	Rho-GEF (DH)	PH
p115-RhoGEF	927		✓	✓	✓
PDZ-RhoGEF	1562	✓	✓	✓	✓
LARG	1544	✓	✓	✓	✓
p63-RhoGEF	580			✓	✓

Table 1.2 Domain Structure of RhoGEF proteins associated with blood pressure. The RhoGEF domain (also known as the Dbl homology [DH] domain), is the catalytically active domain of RhoGEFs and major binding site for RhoA GTPase. Immediately downstream of the RhoGEF domain is a pleckstrin homology (PH) domain, which works cooperatively with the RhoGEF domain to fully activate the RhoA GTPase. Other functional domains in these RhoGEFs are the Regulators of G protein signaling homology (RH) domain and the postsynaptic density 95, disk large, zona occludens-1 (PDZ) domain. Potential blood pressure therapeutics are being developed to target the interaction interface between RhoGEF domain and RhoA. Length is shown in amino acids (AA).

GAP Protein	Length (AA)	Domain							
		BAR	PH	GBD	FF	PBR	GBD	Rho-GAP	SH3
GRAF3	874	✓	✓					✓	✓
P190-RhoGAP	1499			✓	✓✓✓✓	✓	✓	✓	

Table 1.3 Domain Structure of RhoGAP proteins associated with blood pressure. The GAP domain is the catalytically active domain of RhoGAPs. GRAF3 also contains a Bin/amphiphysin/RVS (BAR) domain, a pleckstrin homology (PH) domain, and a SRC homology 3 (SH3) domain, which sense and induce membrane curvature, aid in lipid binding, and promote protein-protein interactions, respectively. In addition to the catalytic GAP domain, p190-RhoGAP also contains a GTP-binding domain (GBD), four diphenylalanine motifs (FF), and a polybasic region (PBR). These regions aid in GTP binding, assist in protein-protein binding, and allow for membrane association, respectively. Potential blood pressure therapeutics involving these RhoGAPs focus on activating the GAP domain. Length is shown in amino acids (AA).

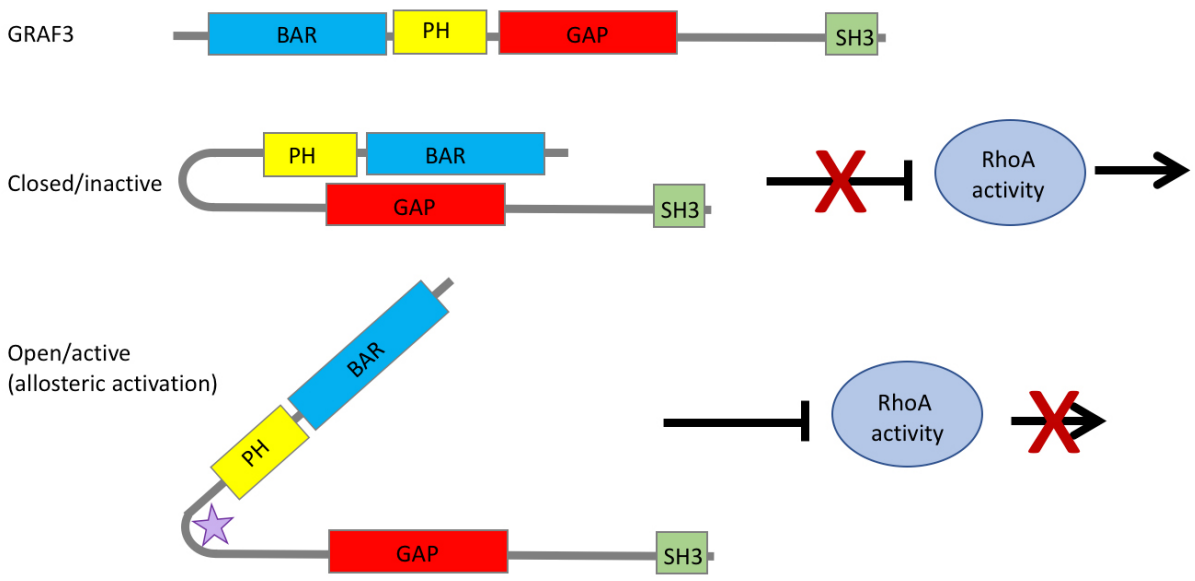


Figure 1.2 Domain Structure and therapeutic strategy for targeting the SMC-specific RhoGAP GRAF3. Like other BAR-PH-GAP containing proteins, the BAR and PH domains of GRAF3 act as a functional unit to autoinhibit the GAP domain, preventing GRAF3's interaction with RhoA. One potential therapeutic strategy is to use allosteric activation (either post-translational modifications or small molecules, represented here by the purple star) to lock GRAF3 in its open conformation, enhancing hydrolysis of GTP to GDP, thereby decreasing the activity of RhoA and promoting SMC relaxation and decreased blood pressure.

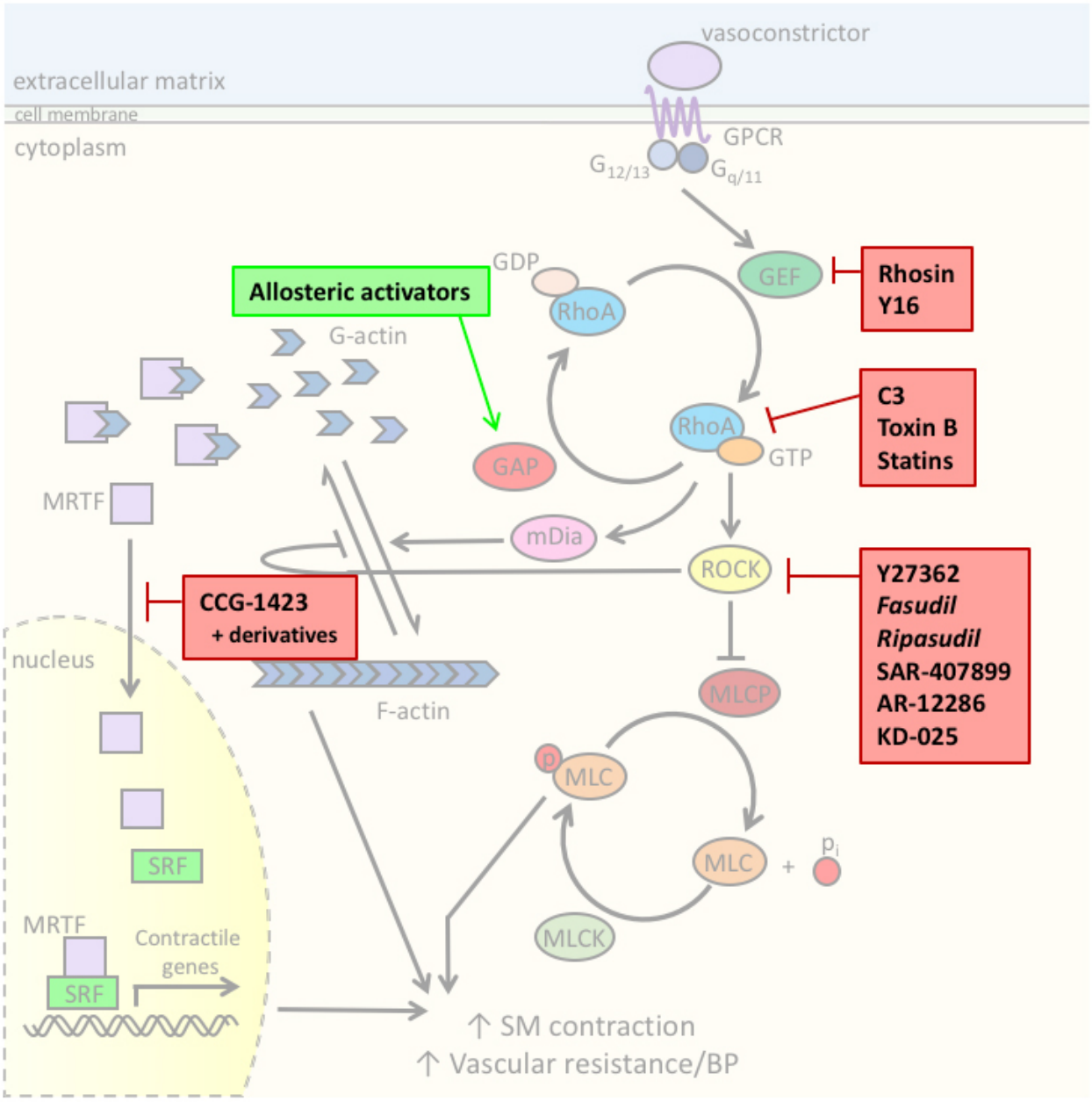


Figure 1.3 Therapeutic potential of the RhoA pathway to treat HTN. There are many components of the SMC RhoA contractile pathway that could be targeted to reduce systemic blood pressure. While not an exhaustive list, shown here in bold boxes are compounds discussed in this article, including those that are proof-of-concept, in clinical development, or currently used in the clinic (italicized).

CHAPTER 2: STRETCH ACTIVATION OF SRC AND FAK KINASES PHOSPHORYLATE GRAF3 AT Y376 IN VASCULAR SMOOTH MUSCLE CELLS TO PROMOTE RELAXATION AND LOWER BLOOD PRESSURE[2]

2.1 Introduction

High blood pressure, known clinically as hypertension (HTN), is a highly prevalent and relevant disease in the Western world and is a major risk factor for myocardial infarction, stroke, and kidney failure. It is estimated that 1 in every 3 adults in the US has HTN and another 1 in 3 has prehypertension[209]. Despite the wide-spread availability of antihypertensives, it is estimated that only half of medicated adults have their blood pressure (BP) under control[210] . One reason for this treatment insufficiency is the complexity of BP homeostasis. Blood pressure regulation involves the integrated control of many different organ systems and the interplay of many genes[24, 28]. One of the key components of HTN, however, is increased peripheral vascular resistance due to the contraction of vascular smooth muscle around arterioles[24, 25, 27, 28]. Smooth muscle cell (SMC) contractility is enhanced by activation of the small GTPase RhoA, which plays a role in phosphorylation of myosin light chain (MLC), stress fiber formation, actin dynamics, and SMC gene expression[29, 211, 212]. Because RhoA's involvement in all of these processes, RhoA has been implicated in the development of HTN[30, 125, 213].

Small GTPases such as RhoA are molecular switches, controlled by GEFs and GAPs. Guanine nucleotide exchange factors (GEFs) turn RhoA "on" by dissociating GDP so free GTP

[2] This chapter contains text, data, and figures being prepared for publication as an original research article and is co-authored by Rachel A. Dee, Xue Bai, Christopher P. Mack, and Joan M. Taylor

can bind while GTPase activating proteins (GAPs) turn RhoA "off" by helping coordinate hydrolysis of GTP. Our lab identified a smooth muscle-specific RhoA GAP protein, called GRAF3 (also known as ARHGAP42), that is capable of regulating vascular tone[36-38, 214]. GRAF3-deficient mice exhibit significant hypertension and increased pressor responses to Angiotensin II and endothelin 1; an effect that can be prevented by treatment with the Rho kinase inhibitor Y-27632[36]. Both large and small arteries from GRAF3-deficient mice also exhibited increased contractility *in vitro* and *in vivo*[36]. Recent GWAS and genetic studies have also identified a role for GRAF3 in BP regulation[15, 16, 38] and recently, a family was identified with a translocation that results in truncated GRAF3[215]. This haploinsufficiency of GRAF3 is associated with HTN and obesity[215]. While there is a clear link between GRAF3 and BP homeostasis, the precise biochemical mechanism of how GRAF3 functions within SMCs is still unclear.

Recently, it was shown that phosphorylation of GRAF3 can cause migration changes and focal adhesion recruitment in fibroblasts[208]. Because GRAF3 is only expressed endogenously in smooth muscle cells, it is necessary to determine its mechanism in this precise cell type. The goal of this study was to identify molecular mechanisms of GRAF3 activity in vascular smooth muscle. Herein we tested the ability for smooth-muscle specific GRAF3 overexpression to reduce basal BP in mice.

Importantly, we found that GRAF3 has an autoinhibitory mechanism of regulation where GRAF3's BAR-PH domains regulate the activity of GRAF3's GAP domain. Furthermore, we found that GRAF3 is phosphorylated by Src or FAK kinases at Y376 in vitro. We also found that this phosphorylation results in a decrease in pMLC in SMC, which may explain the decreases in blood pressure that GRAF3 overexpressing mice experience. These

findings underscore the importance of GRAF3 in blood pressure regulation and lays the foundation for GRAF3 targeted antihypertensive therapies.

2.2 Results

2.2.1 Smooth muscle-specific GRAF3 overexpression decreases systolic blood pressure

Previously, it has been shown that endogenous GRAF3 limits RhoA-dependent SMC tone and that its expression is necessary to maintain normal blood pressure homeostasis; GRAF3 deficient mice exhibit a 25mmHg increase in blood pressure[36]. Strong *in vitro* evidence also shows that ectopic expression of GRAF3 in Wt cultured SMC led to a marked reduction in SMC contractility as assessed by decreased actin polymerization and weakened connections between the cytoskeleton and extracellular matrix. Thus, we sought to test the exciting possibility that elevated SMC-restricted GRAF3 overexpression will lower basal blood pressure and prevent HTN in mice with clinically relevant hypertensive disease.

To this end, we created a novel mouse model in which GRAF3 could be temporally and spatially controlled in a Cre-dependent fashion. Initially we overexpressed WT GRAF3 in the heart during development using constitutively active Nkx2.5 Cre, but this yielded no viable offspring (data not shown). In accordance with other studies, we observed that limiting RhoA activity too much with WT GRAF3 overexpression in cell culture caused cell death[216]; therefore, we created a second Cre-inducible GRAF3 transgene (called $GRAF3^{RQ}$) containing a mutated arginine (R417Q) that exhibits ~40% less GAP activity than WT. To make $GRAF3^{RQ}$ overexpression smooth muscle specific, we crossed the *$GRAF3^{RQ}$* mice to tamoxifen-inducible *SM MHC $CreER^{T2}$* mice (**Figure 2.1A** and **2.1B**).

Using radiotelemetry, we observed blood pressure in 12-16 week old *GRAF3^{RQ}* and *GRAF3^{RQ} SM MHC CreER^{T2}* male mice. Basally, both groups had an average systolic BP of about 125 mmHg (**Figure 2.1C**). After tamoxifen injection and induction of GRAF3^{RQ} overexpression in smooth muscle cells, basal blood pressure for the *GRAF3^{RQ} SM MHC CreER^{T2}* mice significantly decreased by about 10 mmHg compared to experimental controls ($p<0.05$) (**Figure 2.1C**). While GRAF3^{RQ} overexpressing mice were still amenable to BP increases when challenged with the hypertensive agonist L-NAME, importantly, GRAF3^{RQ} overexpressing mice maintained the 10 mmHg decrease compared to their non-overexpressing counterparts ($p<0.05$ for differences in BP each day between the GRAF3^{RQ} overexpressing and non-overexpressing groups, $p=0.1137$ for the differences in the rate of rise of BP between the two groups) (**Figure 2.1C**). Diastolic BP, mean arterial pressure, and heart rate between the two groups did not vary significantly throughout the experiment (**Supplemental Figure S2.1**). This finding supports the hypothesis that enhancing GRAF3 activity could be a novel effective therapeutic for HTN.

2.2.2 GRAF3 displays autoinhibition of its GAP domain by its own BAR-PH domain

In order to assess the plausibility of a GRAF3 targeted therapeutic, it is necessary to understand the molecular mechanism of GRAF3 within SMCs. GRAF3 contains BAR, PH, GAP, and SH3 domains (**Figure 2.2A**), each of which may alter GRAF3's subcellular location, cellular interactions, and function[217, 218]. To better understand how these domains interact with one another, we used PyMol and ClusPro to build a predictive molecular model of GRAF3. Because the crystal structure of GRAF3 is currently unsolved, we used the BAR-PH domain from Appl1[219] and the GAP domain from GRAF1[220] to build our model. Appl1 is a Rab5 effector protein involved in cell proliferation and endosomal trafficking signaling pathways[221, 222]

while GRAF1, as mentioned previously, is the founding member of the GRAF family, involved in integrin and endocytic signaling[223, 224]. The domains from these proteins were chosen because of their structural similarities to GRAF3 and their high conservation of functionally important or interface interacting residues. The experimental structures of both Appl1 (BAR PH) and GRAF1 (GAP) are available on the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) (www.rcsb.org)[222]. We then performed a molecular docking search using ClusPro to dock GAP domains onto the BAR-PH domain dimer with a distance less than 30Å. Possible docking modes recovered 17 solutions that clustered into 2 groups of structures (**Figure 2.2B** and **2.2C**). The structure on the top (**Figure 2.2B**) likely represents an open 'active' state because all of the residues important for GTPase binding and GAP activity (pink and dark blue, respectively) are exposed. The structure on the bottom (**Figure 2.2C**) represents a closed, 'inactive' conformation since the conserved face of the GAP domain (yellow) is lying on the BAR-PH domain (dark green and light green or dark purple and light purple) effectively masking the residues required for GTP hydrolysis. Interestingly, the change in orientation of the GAP domain between the two dockings is a simple rotation about the horizontal axis by 90 degrees (note the different positions of the teal colored residues). Importantly, our analysis suggests that the GAP domain interacts with the convex surface of the banana-shaped BAR dimer, a model consistent with biochemical evidence that the BAR domain of (similarly structured) oligophrenin simultaneously interacts with membranes, but through positive charged lysines that lie on the opposite concave surface[206].

Other BAR-PH-GAP-containing proteins such as oligiophrenin and GRAF1 display autoinhibition between the BAR-PH domain and GAP domain with high specificity[206]. Our model supports the idea that the BAR-PH domain of GRAF3 could autoinhibit its own GAP

domain in a similar fashion. To test this, we expressed full length Myc-GRAF3 in Rat Aortic SMCs (RaAoSMC) and saw attenuation of actin stress fiber formation, which is indicative of inhibition of RhoA, and therefore active GRAF3 (**Figure 2.2D**). When we co-expressed excess Flag-GRAF3 BAR-PH alongside the full-length Myc-GRAF3, this effect was blocked, signifying more active RhoA (and thereby less active GRAF3); Accordingly, the excess GRAF3 BAR-PH domain was able to inhibit the activity of the GAP domain of GRAF3 (**Figure 2.2D** and **2.2E**).

2.2.3 GRAF3 is phosphorylated at Y376 by Src and FAK kinases in vitro

Our molecular model and NetPhos analysis predict that GRAF3's autoinhibition could be controlled by a post-translational modification, such as phosphorylation, and may allow GRAF3 to toggle between active and inactive (autoinhibited) states[225, 226]. Src and Focal Adhesion Kinase (FAK) are two non-receptor tyrosine kinases we hypothesized could phosphorylate GRAF3 and cause this shift in activation. Both Src and FAK are involved in outside-in cellular communication and are activated by mechanical stretch and integrin signaling[25, 227-229]. Because of the continuous stretch and relaxation of the arteries as blood pumps through the body, vascular smooth muscle cells are consistently exposed to mechanical stretch and its concomitant signaling cascades. It has been shown that signaling through Src and FAK not only affects Rho family activation[230], but Src and FAK also interact with other members of the GRAF family (the founding member of the GRAF family, GRAF1, was named for its association with FAK; GTPase Regulator Associated with Focal Adhesion Kinase)[197-200]. Previous large-scale phosphoproteomic studies identified tyrosine residues, 376 and 792, as potential phosphorylation sites by Src and FAK[208, 231]. Our molecular model predicts that Y376 is on the outside of the

folded GRAF3 protein in the unstructured linker region between the PH and GAP domains (between the red and orange residues), a location that could be susceptible to phosphorylation (**Figure 2.3A**). Tyrosine 792 is located in a proline/serine rich region just upstream of the SH3 domain, which is not shown in our model.

To test whether Src or FAK were capable of phosphorylating GRAF3 at these sites, we performed immunoprecipitation and radioactive kinase assay experiments. Immunoprecipitation of Myc-tagged GRAF3 from Cos cells co-expressing Src or superFAK (a variant of FAK with increased catalytic activity[232]) show significantly decreased tyrosine phosphorylation when GRAF3 has a phospho-deficient mutation at Y376 (Y376F) (**Figure 2.3B** and **2.3C,** respectively). Phospho-deficient mutation of Y792 caused little change in phospho-tyrosine signal (**Supplemental Figure 2.2**), indicating that Y376 is the more dominant site of phosphorylation, thus we only focused on Y376 for the remainder of the study. The importance of this site (Y376) was validated by probing immunoprecipitates with a phospho-specific GRAF3 Y376 antibody (**Figure 2.3F** and **2.3G**). Again, tyrosine phosphorylation was significantly decreased in cells containing the Y376F phospho-deficient variant of GRAF3 (**Figure 2.3F** and **2.3G**). To ensure that phosphorylation of GRAF3 was caused by Src and FAK directly and not by an intermediary kinase within cells, we performed radioactive kinase assays, where purified GRAF3 BAR-PH-GAP or purified GRAF3 BAR-PH-GAP Y376F were incubated with active purified Src or FAK and ATP γP^{32}. Radioactive phosphates were incorporated into WT GRAF3 when incubated with either Src or FAK and this radioactive phosphate signal decreased when Src or FAK were incubated with GRAF3-BAR-PH-GAP Y376F (**Figure 2.3D** and **2.3E**). Therefore we concluded that both Src and FAK can phosphorylate GRAF3 at Y376 in cells.

2.2.4 Phosphorylation of GRAF3 at Y376 increases GAP activity and decreases RhoA activity in smooth muscle cells

Until now, no one has shown the relevance and function of GRAF3 phosphorylation at Y376 in smooth muscle cells. Our working model predicts that phosphorylation of GRAF3 at Y376 could cause a change in GAP activity. Indeed, TR-FRET GAP assays showed that the phospho-mimetic GRAF3 Y376E has more GAP activity than WT GRAF3 and more rapidly converts GTP to GDP (**Figure 2.4A, 2.4B,** and **2.4C**). This increase in GAP activity is physiologically relevant in SMC. When RhoA signaling is downregulated, Rac and Cdc42 signaling are upregulated, leading to increased lamellipodial formation and "arborization" of SMC. Analyzing the cell morphology of RaAoSMC transfected with GRAF3 variants revealed that transfection of SMC with the Y376E variant decreases the percent of arborized cells compared to WT, while transfection of RaAoSMC with the Y376F phospho-deficient variant increases the percent of arborized cells compared to WT (**Figure 2.4D** and **2.4E**). RaAoSMC expressing a delta-BAR variant of GRAF3 had an even greater decrease in the amount of arborized cells compared to WT or the Y376E phosphor-mimetic, further supporting the autoinhibition mechanism of the GAP domain by the BAR-PH domains (**Figure 2.4E**).

2.2.5 GRAF3 phosphorylation at Y376 prevents phosphorylation of myosin light chain in smooth muscle cells

It's important to understand how this altered GRAF3 and RhoA activity will effect downstream contraction of SMC in a physiologically relevant context. Compared to SMC infected with GFP, RaAoSMC infected with Flag-GRAF3 lentivirus exhibited a decrease in

phosphorylation of myosin light-chain (pMLC), the major determinant of smooth muscle contraction (**Figure 2.5**). Infection of SMC with Flag-GRAF3 Y376F resulted in a near-normal amounts of pMLC (**Figure 2.5**).

2.3 Discussion

Previously, we have shown that the smooth-muscle specific RhoA GAP protein GRAF3 is involved in blood pressure homeostasis [36-38]. The current study further supports this by demonstrating that increased expression of GRAF3 in smooth muscle cells leads to decreased systolic blood pressure in mice and that increased activity of GRAF3 in SMCs caused by stretch leads to a decrease in pMLC via phosphorylation activation of GRAF3 at Y376 by Src and FAK (**Figure 2.6**).

Excitingly, we found that GRAF3^{RQ} overexpression in SMC could significantly reduce basal systolic BP in mice and maintain this difference through L-NAME induced HTN. Albeit not tested in this study, this suggests that overexpressing GRAF3 *after* the development of HTN (which more accurately mimics human disease) would also effectively lower BP. While L-NAME recapitulates volume overload, a common characteristic in human HTN, it is of future interest to test the widespread ability of GRAF3 overexpression to prevent or reverse BP increases in other models of essential HTN that imitate different features also found in human HTN[233]. As evidenced by the embryonic lethality of our mice constitutively overexpressing GRAF3 in their hearts and our observation that too much GRAF3 (and thereby too little RhoA activity) in cultured cells can lead to cell death, we conclude that there is a fine balance of GRAF3/RhoA activity required for survival and appropriate tone of SMCs in the vasculature.

While our data support Y376 as the main site of GRAF3 tyrosine phosphorylation, both our phospho-tyrosine immunoprecipitations and radioactive kinase assay data indicate that GRAF3 may be phosphorylated by Src and (more so by) FAK at other sites besides Y376. This is corroborated by the PhoshoSitePlus database, which reports potential post-translational modifications of proteins from large mass-spectrometry studies (phosphosite.org). Tyrosines 376 and 792 were identified as sites of post-translational phosphorylation in over 200 and 600 independent studies, respectively, however at least 2 other tyrosines were identified in at least 1 study each and 19 other residues were identified as potential sites of phosphorylation[231]. While both Y76 and 792 are conserved between multiple species[231], we show that Y376, located between the PH and GAP domains can control GAP function of GRAF3. Tyrosine 792 is located in the proline/serine rich region a short ways upstream of the SH3 domain (**Supplemental Figure S2.2**), and thus more likely affects activities of GRAF3's SH3 domain, such as intracellular localization and protein-protein interactions, though we did not test this in our current study[208].

Previously, we've shown that GRAF3 depletion affects pMLC and contraction *in vivo*. GRAF3-deficient mice had more pMLC staining in SMC-heavy tissues such as the stomach, than their WT counterparts[36]. In this study, we saw a decrease in pMLC in SMC that overexpress activated GRAF3 *in vitro*. It will be important to determine if the levels of GRAF3 pY376 activation occur *in vivo*, in tissue samples from animals subjected to hypertension or *ex vivo* from isolated muscle segments subjected to stretch.

Arteries are continuously exposed to pulsatile pressure as blood flows throughout the body after every heartbeat. To protect blood vessels from injury, vascular SMC have an intrinsic myogenic response, contracting when there is an increase in pressure and dilating when there is a

decrease in pressure. Detecting and transducing these mechanical signals, therefore, is a key signaling mechanism for vascular SMC and we postulate that the GRAF3 activation mechanism discovered in this study is part of a mechanotransduction feedback loop to keep RhoA activity and SMC contractility constrained.

Mechanical stretch signals activate ion gated channels, cell adhesion molecule proteins, and integrins on the surface of SMCs which lead to upregulation of the PI3K, ERK, and RhoA pathways to control cell proliferation, survival, motility and migration, and contractility. These pathways lead to the Ca^{2+}-calmodulin dependent phosphorylation of myosin light chain kinase, which phosphorylates myosin light chain (MLC), allowing MLC to interact with actin and form cross-bridges and initiate contraction. Simultaneously, these pathways activate RhoA, which stimulates Rho kinase (ROCK). ROCK inhibition of myosin light chain phosphatase further enhances contraction of SMCs. RhoA also promotes the polymerization of F-actin from G-actin, releasing G-actin bound myocardin related transcription factors (MRTFs) that bind to serum response factor (SRF), translocate to the nucleus, and promote transcription of SMC contractile genes such as myosin heavy chain, SM22, calponin, and SM alpha-actin. The MRTF/SRF localization to the nucleus also increases the expression of GRAF3[37], in a feedback loop to level off RhoA activity and keep contractile gene expression within a certain range[37]. We postulate that stretch-induced integrin activation of FAK and Src feed into this loop, by phosphorylating GRAF3 at Y376, thereby activating existing and freshly transcribed GRAF3 to reduce RhoA activity, and restrict contractile gene expression to within a certain range.

This feedback loop and contractile protein level stabilization is paramount to the phenotypic modulation of SMC. Smooth muscle cells do not terminally differentiate; they retain remarkable plasticity and can switch between a contractile phenotype and a proliferative

phenotype, depending on the level of contractile gene expression. Enhancement of the contractile phenotype leads to stiffening of SMC and large artery stiffening. Preclinical studies have suggested that arterial stiffening is a precursor to HTN and other CVD outcomes[234], but there are no approved therapies that directly target arterial stiffening. Studies in spontaneously HTN rats have also linked the ROCK/SRF/MRTF pathway to control of arterial stiffening. Therefore, long-term activation of GRAF3 and dampening of SMC contractile gene profiles could help prevent arterial stiffening and significantly slow the progression of HTN, especially in ageing populations that are prone to arteriosclerosis.

In conclusion, HTN is one of the leading global health risks[235], but the complexity of BP homeostasis makes HTN a difficult disease to treat. Our study provides a mechanistic link between phosphorylation of GRAF3 at Y376 and the relaxation of SMC, leading to decreased BP and we argue this could also contribute to the prevention of arterial stiffening. Our findings therefore support the development of novel GRAF3-targeted therapies to treat HTN and CVD.

2.4 Materials And Methods

Generation and characterization of GRAF3^{RQ} SMMHC-CreER^{T2} mice

Chimeric mice were produced in-house by injection of *GRAF3^{RQ}* ES cells into the blastocoel cavity of mouse blastocysts by standard procedures. The *GRAF3^{RQ}* strain was established using two independent chimeras that demonstrated germline transmission when bred to wild-type C57bl6 mice. *GRAF3^{RQ} SMMHC-CreER^{T2}* mice were generated by crossing *GRAF3^{RQ}* female mice with *SMMHC-CreER^{T2}* male mice. All experiments were performed using age and sex-matched genetic controls. Only male mice were used in this study because the

SMMHC-CreER^{T2} is located on the Y chromosome. Genotyping was performed using DNA isolated from tail biopsies using locus-specific primers (for *GRAF3^{RQ}*: 5′-gttcggcttctggcgtgtgac; 3′- ggtccctcgaagaggttcactag, for *SMMHC-CreER^{T2}*: 5'-tgaccccatctcttcactcc; 5'-aactccacgaccacctcatc; 3'-agtccctcacatcctcaggtt). Overexpression was assessed by comparing levels of myc-tagged GRAF3 in organ lysates from tamoxifen (100mg/kg for 3 days via oral gavage) or non-tamoxifen treated animals. Animal husbandry was provided by staff within the University of North Carolina Division of Comparative Medicine and all animal procedures were approved by our accredited American Association for Accreditation of Laboratory Animal Care committee and the Institutional Animal Care and Use Committee.

Blood Pressure Measurements

Conscious blood pressure was measured in male mice aged 12–16 weeks using radiotelemetry (Data Sciences International). Implantable mouse BP transmitters (PA-C10) were used to record arterial pressure in conscious and freely moving mice. In brief, the mice were anaesthetized with 2% isoflurane, the telemetry catheter was inserted into the left carotid artery of the mouse and the catheter tip was advanced into the thoracic aorta. The catheter was fixed in the left carotid artery and the transmitter was inserted subcutaneously along the right flank. Mice were allowed 7 days of recovery following transmitter implantation and were housed individually in a standard polypropylene cage placed on a radio receiver. Following baseline readings, mice were treated with tamoxifen (100mg/kg) for 3 consecutive days via oral gavage. Increasing doses of Nω-Nitro-L-arginine methyl ester hydrochloride

(L-NAME salt) (50mg/L, 150mg/L, 450mg/L) were added to drinking water for 7 days (per dose). Mice were maintained in a 12:12-h light–dark cycle. All blood pressure parameters were telemetrically recorded and stored with the Ponemah data acquisition system (Data Sciences International). Recordings were collected for 5 minutes every 30 minutes throughout the study and averaged over a 24 hour period for each day.

Cell culture and transfection

Cos cells and RaAoSMC were maintained in DMEM or DMEM-F12 media, respectively, supplemented with 10% fetal bovine serum and 0.5% penicillin/streptomycin. Cells were transfected with plasmids using Trans-IT (Mirus Bio) transfection reagents according to manufacturer's protocol. Myc-GRAF3 was made by cloning into a pCMV-Myc vector (Clonetech). Flag-GRAF3 Bar-PH was made by cloning into a pcDNA3 vector. GST-GRAF3-BAR-PH-GAP was made by in-fusion cloning (Clonetech) into a pGEX6.1 vector (GE). All phospho-mimetic and phospho-deficient mutations were made by site-directed mutagenesis (Clonetech).

Molecular Modeling

Molecular models of GRAF3 were built using PyMol to combine the BAR-PH domains of Appl1 (PDB ID 2Q13, Human Appl1) and the GAP domain of GRAF1(PDB ID 1F7C, chicken GRAF1). The domains from these proteins were chosen because they were the most similar and highly conserved proteins/domains (compared to GRAF3) that had solved experimental structures available on the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) (www.rcsb.org). The GAP domain was then docked onto the BAR-PH

domain using the Clus Pro protein-protein docking server[236-238]. Models were narrowed down by analyzing the location and plausibility of residues important for GTPase binding and hydrolysis.

Western blotting

Cells were lysed in RIPA buffer + 0.5% Triton with protease and phosphatase inhibitors. Protein concentration was determined by using a colorimetric BCA assay (Pierce). Lysates were electrophoresed on 10-15% SDS-PAGE gels, transferred to PVDF membrane and immunoblotted with specific antibodies as indicated using a 1:1000 dilution. Myc, Src, pMLC, MLC, and Arhgap42 pY376 antibodies were from Cell Signaling; Flag antibody from Sigma; pTyr antibody from EMD Milipore; GAPDH from Novus Biologicals; GFP antibody from Meridian Life Sciences. Blots were washed in TBST (TBS plus 0.05% Triton X), followed by incubation with horseradish peroxidase conjugated secondary antibodies (Amersham) at a 1:2,000 dilution. Blots were visualized after incubation with chemiluminscence reagents (ECL, Amersham).

Immunoprecipitation

Cos cells were transfected with myc-GRAF3 variants and either Src or Flag-superFAK plasmid constructs. After 24 hours, cells were lysed with RIPA buffer + 0.5% Triton and myc-tagged GRAF3 was immunoprecipitated overnight from cell lysate using a myc antibody (Cell Signaling) conjugated to Dynabeads Protein G (Invitrogen). Immunoprecipitates and lysates were electrophoresed on a 10% SDS-PAGE gel and immunoblotted with indicated antibodies.

Protein Purification

GST-GRAF3 constructs were transformed into BL-21 competent *E. coli* and expressed by IPTG induction at 16°C overnight. *E.coli* were lysed with a lysozyme-buffer (20mM HEPES, 150mM NaCl, 5mL MgCl2, 1% Triton X-100, 1mM DTT, 5mg/mL lysozyme, protease inhibitors), sonicated, and then protein was purified using glutathione-sepharose beads (GE Life Sciences). For GAP assay, constructs were dissociated from beads via reduced glutathione and GST was removed via PreScission Protease (GE LifeSciences) according to manufacturer's protocol.

Radioactive In Vitro Kinase assay

Purified GST-GRAF3-BAR-PH-GAP or GST-GRAF3 BAR-PH-GAP-Y376F were incubated in kinase buffer (25mM MOPS, 25mM MgCl2, 5mM EGTA, 2mM EDTA, 12.5mM β-glycero-phosphate, 2.5 mM DTT) with either active FAK (R&D Systems) or Src (Sigma) kinase and radioactive γP^{32} (Perkin Elmer) at 30°C for 15 minutes. Reaction mixture was run on a 10% SDS-PAGE gel, gel was dried, and phosphorylation was assessed by radiograph. Gel was rehydrated and stained with Coomassie Blue to assess total protein concentration.

TR-FRET GAP assay

The GAP activity of the GRAF3 BAR-PH-GAP proteins were measured using the Transcreener Time Resolved-Fluorescence Resonance Energy Transfer (TR-FRET) GDP assay kit (BellBrook Labs). Briefly, various concentrations of purified WT or Y376E mutated GST-GRAF3 BAR-PH-GAP were combined with RhoA , GTP, and GDP HiLyte647 tracer (bound to a GDP antibody conjugated to terbium) as per manufacturer's instructions. Amount of FRET signal was

measured over 140 minutes on a CLARIOstar (BMG Labtech) plate reader ($Ex_{320}/Em_{615}/Em_{665}$).

Morphology Assay

RaAoSMC were transfected with myc-GRAF3 variants for 24hours, fixed in 4% PFA, permeabilized with 0.5% Triton X-100 and stained with 1:500 myc (Cell Signaling) and 1:500 Alexaflour 488 Goat anti-mouse (Invitrogen) antibodies. Thirty 20x image tiles were taken on an Olympus IX81 microscope and then stitched together in Image J software. Transfected cells of each morphology type were counted using the Image J cell counter.

Lentiviral Infection

GFP and Flag-GRAF3 (WT or phosphomimetic and phosphodeficient variants) lentiviruses were generated by transient transfection of HEK293T cells with a lentiviral vector together with a packaging plasmid and an envelope plasmid (PSPAX2 and PMD2G). The supernatant containing lentiviruses was collected, incubated with a 40% PEG solution O/N, and then filtered through a 0.45μM sodium acetate filter, and stored in aliquots at -80°C. RaAoSMC were infected with GFP or Flag-GRAF3 lentivirus with the aid of Polybrene (Sigma). After 48 hours, cells were lysed in 2X Laemmli sample buffer, and subjected to Western Blotting.

Statistics

All data represent at least three individual experiments and are presented as means ± standard deviation. Means were compared by Student's *t*-test or linear regression (where indicated) and statistical significance is reported as *p*-values. Sample sizes were chosen based on an

extensive literature search and standard exclusion criterion of two standard deviations from the mean were applied. Band intensities were quantified using ImageJ software. All statistics were calculated in Excel or GraphPad Prism8.

2.5 Figures

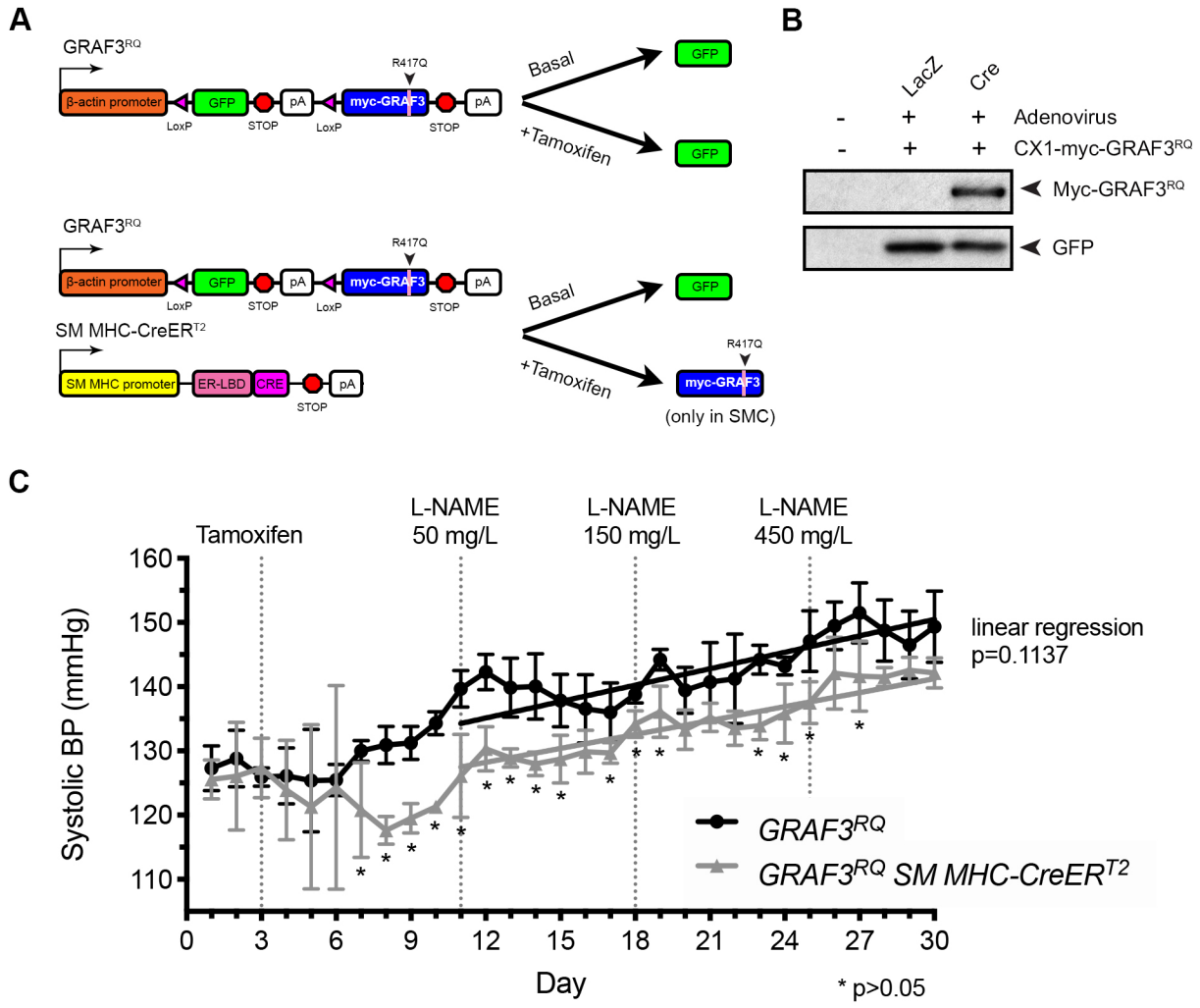


Figure 2.1 SMC-specific GRAF3^{RQ} overexpression decreases basal systolic blood pressure and maintains decrease through L-NAME dependent hypertension. (A) schematic of GRAF3^{RQ} stop and go transgenic construct and tamoxifen-inducible SM MHC-MHC-CreER^{T2} construct used for SMC-specific GRAF3 overexpression mouse experiment. **(B)** Western analysis of Cos cells transfected with the GRAF3^{RQ} plasmid and infected with Cre or LacZ control virus. Data is representative of 3 independent experiments. **(C)** Average 24 hour systolic blood pressure, measured via radio-telemetry, of unrestrained, conscious GRAF3^{RQ} and GRAF3^{RQ} SM MHC-CreER^{T2} mice before and after tamoxifen treatment (100mg/kg for 3 consecutive days) and increasing L-NAME doses (50mg/L, 150mg/L, or 450mg/L) given for a week (each) in drinking water. Data are expressed as mean ± SD; n=4 for GRAF3^{RQ} mice and n=3 for GRAF3^{RQ} SM MHC-CreER^{T2}.*p>0.05 vs. GRAF3^{RQ} (Student's t-test). Linear regression analysis was performed to compare the slope of the two groups after L-NAME treatment (p=0.1137).

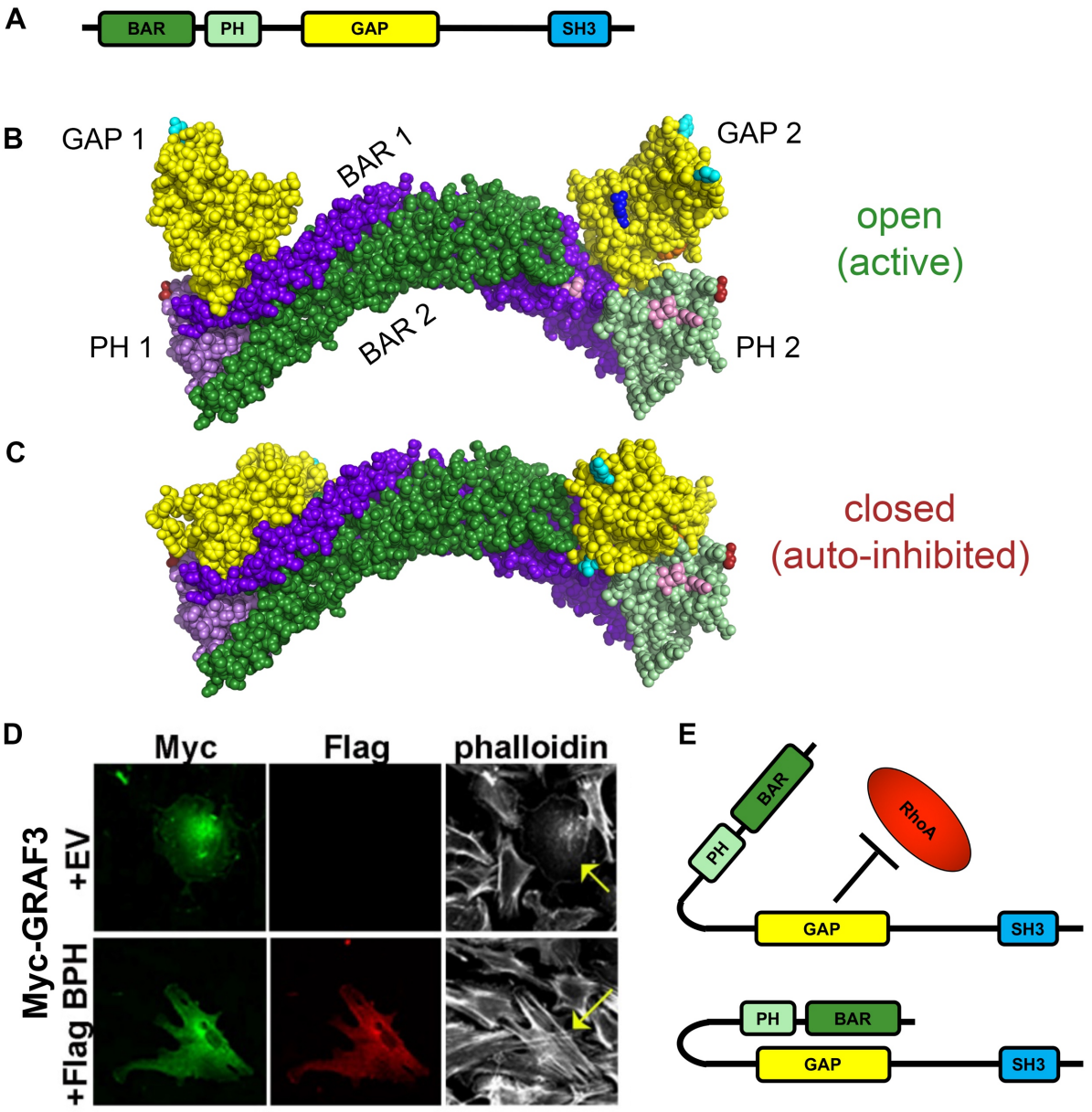
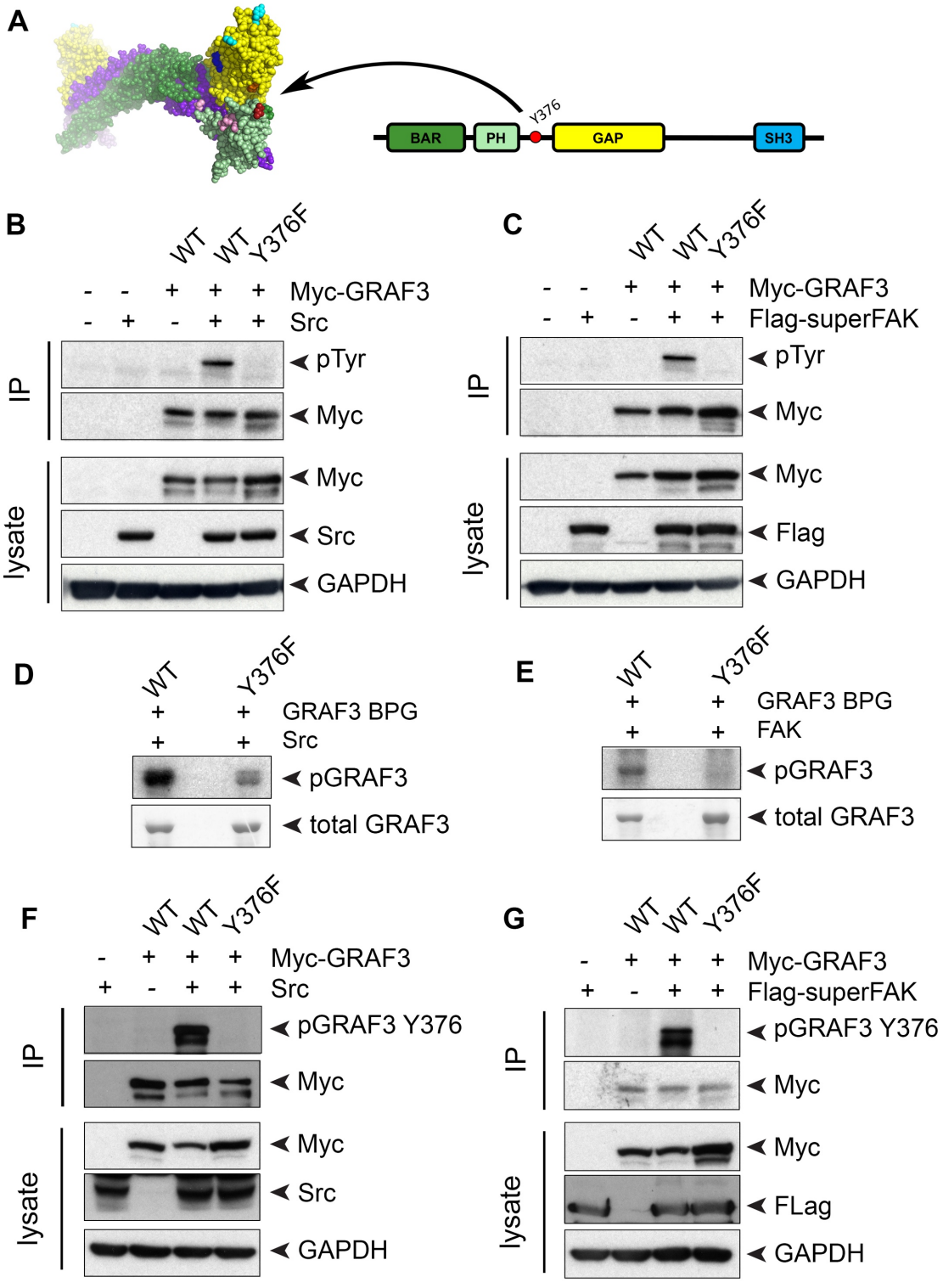


Figure 2.2 BAR-PH mediated autoinhibition of GRAF3. **(A)** Schematic of GRAF3 domain structure. Structures of the GRAF3 BAR-PH-GAP dimer were created using Pymol and the solved, similar structures of Appl1 (BAR-PH) and GRAF1 (GAP). ClusPro docking simulations predicted 2 conformations for GRAF3: an **(B)** open and active or **(C)** closed and auto-inhibited. Color scheme follows: BAR 1 (dark purple), PH 1 (light purple), BAR 2 (dark green), PH 2 (light green), GAP 1 and 2 (yellow), arginine fingers (active site) (dark blue), RhoA docking sites (pink), C-terminus of PH domain (red), N-terminus of GAP domain (orange); residues in teal aids in visualizing rotation. **(D)** Immunofluorescent staining of RaAoSMCs transfected with Myc-GRAF3 alone or Myc-GRAF3 co-expressed with Flag-BAR-PH (BPH) domain. Arrows point to phalloidin-stained cells of interest that are positive for Myc- (green) and/or Flag- (red) staining. **(E)** Schematics of GRAF3 in open, active, or autoinhibited, inactive conformations, which correspond to the phalloidin staining and stress fiber patterns observed in panel **D**.


A
BAR
PH
Y376
GAP
SH3
B
WT
WT
Y376F
Myc-GRAF3
Src
IP
pTyr
Myc
lysate
Myc
Src
GAPDH
C
WT
WT
Y376F
Myc-GRAF3
Flag-superFAK
IP
pTyr
Myc
lysate
Myc
Flag
GAPDH
D
WT
Y376F
GRAF3 BPG
Src
pGRAF3
total GRAF3
E
WT
Y376F
GRAF3 BPG
FAK
pGRAF3
total GRAF3
F
WT
WT
Y376F
Myc-GRAF3
Src
IP
pGRAF3 Y376
Myc
lysate
Myc
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GAPDH
G
WT
WT
Y376F
Myc-GRAF3
Flag-superFAK
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pGRAF3 Y376
Myc
lysate
Myc
FLag
GAPDH

Figure 2.3 Src and FAK kinases phosphorylate GRAF3 at Y376 in vitro (previous page). **(A)** Location of Y376 on GRAF3 schematic indicated by circle between PH and GAP domains. On the 3D model, arrow indicates approximate location, in the unstructured, unmodeled linker between the C-terminus of the PH domain (red) and the N-terminus of the GAP domain (orange). Cos cells were transfected with the indicated Myc-GRAF3 variant and either **(B)** Src or **(C)** superFAK. Myc-tagged GRAF3 was immunoprecipitated from cell lysate and blots were probed for tyrosine phosphorylation, as well as myc, Src or Flag, and GAPDH as controls. Purified GRAF3 BAR-PH-GAP (BPG) domain and GRAF3 BAR-PH-GAP Y376 were subjected to a radioactive kinase assay using activated **(D)** Src or **(E)** FAK and ATP γP^{32}. Phosphorylated and total GRAF3 are shown by radiograph (top) or Coomassie Blue staining (bottom), respectively. Cos cells were transfected with the indicated Myc-GRAF3 variant and either **(F)** Src or **(G)** superFAK. Myc-tagged GRAF3 was immunoprecipitated from cell lysate and blots were probed for phospho-GRAF3 Y376, as well as myc, Src or Flag, and GAPDH as controls.

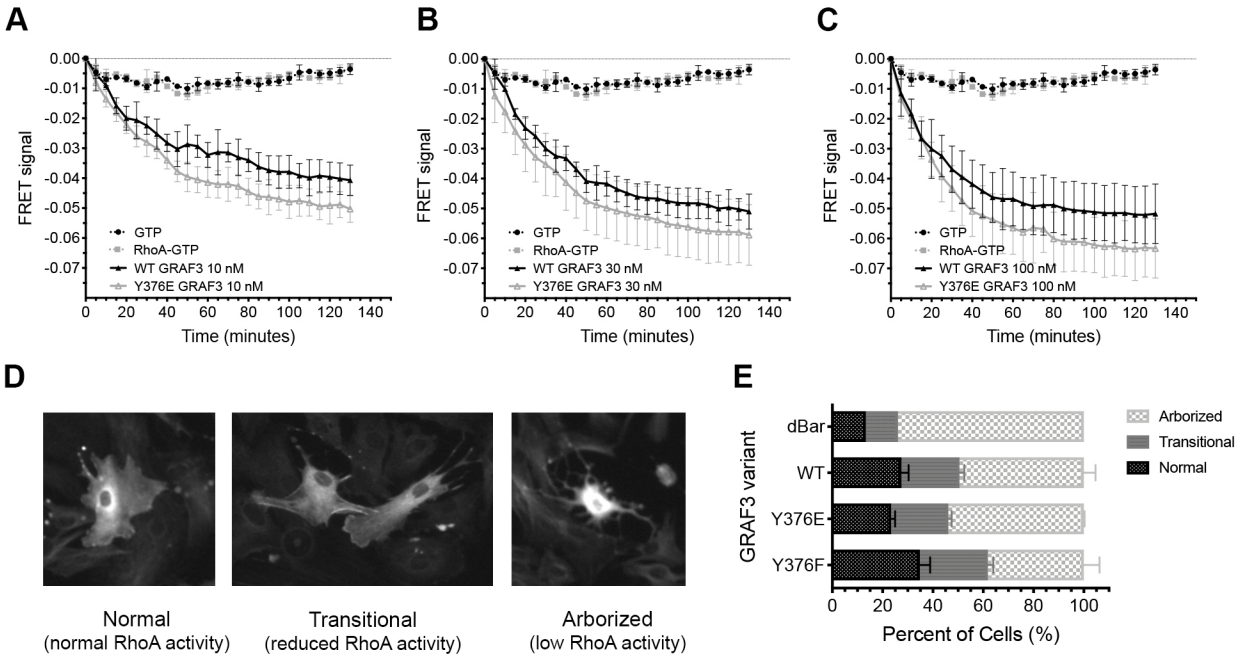


Figure 2.4 Phosphorylation of GRAF3 at Y376 increases GAP activity *in vitro* and decreases RhoA activity in SMC. TR-FRET GDP assay was performed using **(A)** 10nM, **(B)** 30nM, and **(C)** 100nM purified WT or Y376E mutated GRAF3 BAR-PH-GAP. A decrease in signal indicates an increase in GTP to GDP conversion. **(D)** RaAoSMC were transfected with the indicated Myc-GRAF3 variants and stained with Myc and anti-mouse antibodies. Cell morphology of positive cells were classified as normal, transitional, or arborized as a readout of RhoA activity. **(E)** Percent cells in each category were compared between variants. n=150-200 cells per variant. dBar, delta Bar domain.

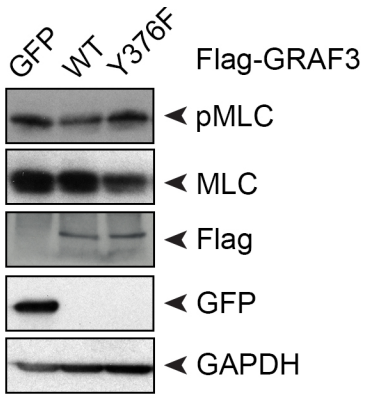


Figure 2.5 GRAF3 phosphorylation at Y376 decreases pMLC in SMC. Representative Western analysis of pMLC in RaAoSMC infected with GFP or Flag-GRAF3 lentivirus variants. Experiment was repeated 3 times (n=3).

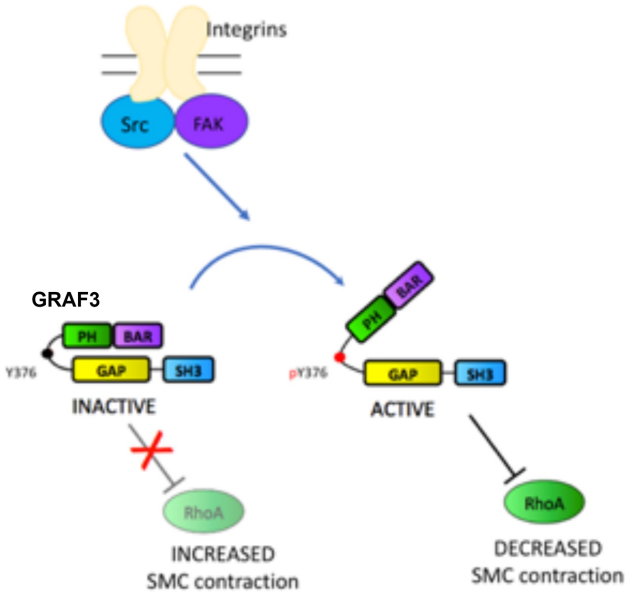
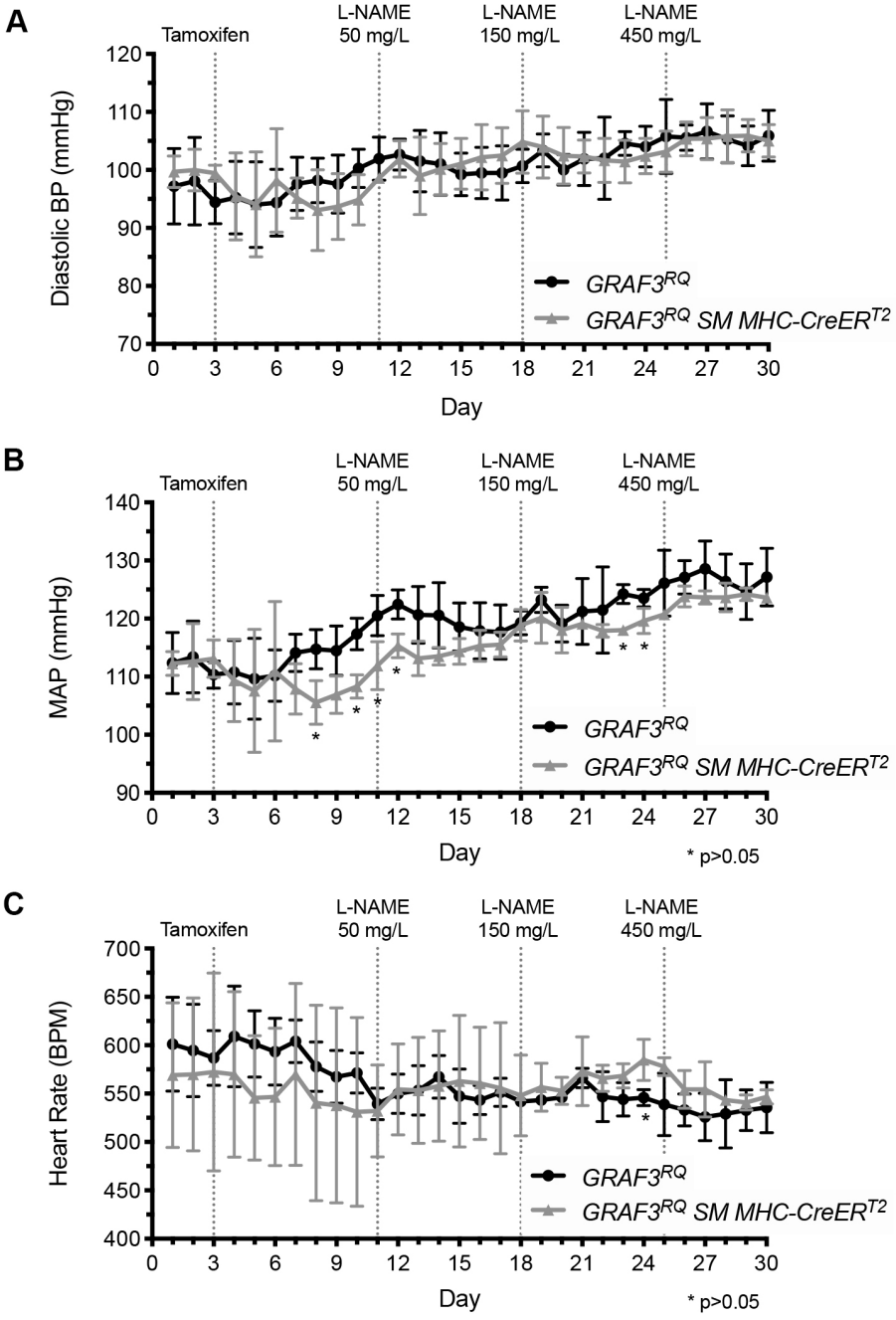
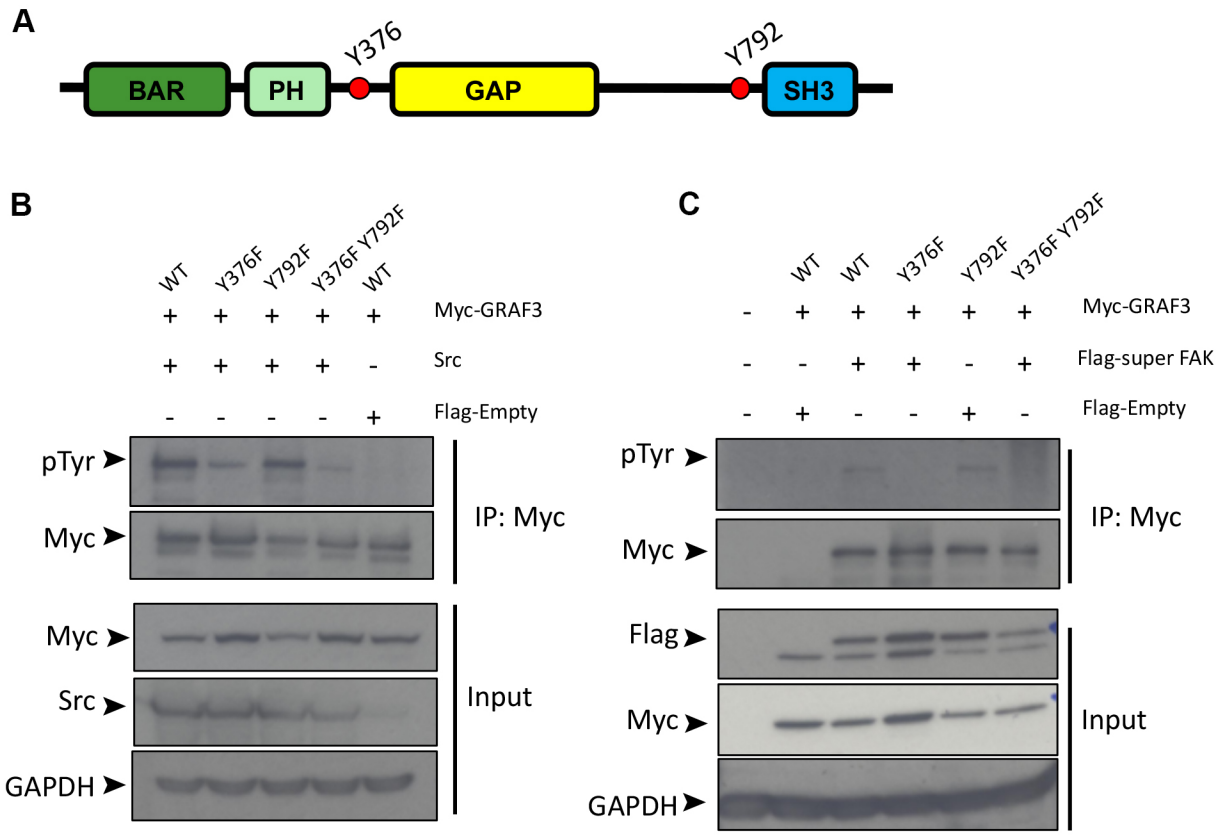


Figure 2.6 Working model of GRAF3 activation. Mechanical signals outside the cell activate integrins along the cell membrane. Integrin signaling in turn activates Src and FAK kinases which phosphorylate GRAF3 at Y376. Phosphorylation at Y376 causes a conformational shift and activation of GRAF3, which inhibits RhoA signaling and results in decreased SMC contraction.

2.6 Supplemental Figures



Supplemental Figure S2.1 SMC-specific GRAF3^{RQ} overexpression has no effect on diastolic BP, MAP, or HR. Average 24hr **(A)** diastolic BP **(B)** MAP, and **(C)** HR measured via radio-telemetry, of unrestrained, conscious *GRAF3^{RQ}* and *GRAF3^{RQ} SM MHC-CreER^{T2}* mice after tamoxifen treatment (100mg/kg for 3 consecutive days) and increasing L-NAME doses (50mg/L, 150mg/L, or 450mg/L) given for a week (each) in drinking water. Data are expressed as mean ± SD; n=4 for *GRAF3^{RQ}* mice and n=3 for *GRAF3^{RQ} SM MHC-CreER^{T2}*.*p>0.05 vs.GRAF3^{RQ} (Student's t-test).



Supplemental Figure S2.2 Phosphorylation of GRAF3 by Src and FAK occurs at Y376 (predominant) and Y792 (to a lesser degree). **(A)** Schematic of GRAF3 domain structure; location of Y376 and Y792 indicated with circles. Cos cells were transfected with the indicated GRAF3 variant and either **(B)** Src or **(C)** superFAK. Myc-tagged GRAF3 was immunoprecipitated from cell lysate and probed for tyrosine phosphorylation. Data is representative of 3 independent experiments (n=3).

CHAPTER 3: CONTRIBUTION OF GRAF2 TO BLOOD PRESSURE REGULATION AND ARTERIAL STIFFNESS[3]

3.1 Introduction

Although HTN is an important risk factor for cardiovascular disease, the precise mechanisms by which it exerts detrimental effects on the cardiovascular system are still unclear. Recent studies have implicated artery stiffness in the pathology of HTN and this parameter has been shown to be a valuable predictor of end organ failure[239-243]. Decreased vessel compliance elevates the mechanical load on the myocardium but also increases peripheral pulse-pressure in the microvasculature resulting in tissue damage in high flow organs such as the brain and kidneys. It is thought that the activation of endothelial cells (EC) and SMC by increased vessel wall stress is an early event that leads to vessel remodeling and atherogenesis[244-246]. In fact it is now recognized that increased vascular stiffness during aging or the development of HTN results from changes in the intrinsic mechanical properties of vSMC[247, 248], in addition to changes in the composition of the vessel's extracellular matrix (i.e. an increased ratio of collagen to elastin).

Previously, our lab found that the RhoA GAP protein GRAF3 plays a role in BP homeostasis, regulating expression of contractile genes and serving as a pressure-sensitive rheostat in SMC. During our qPCR-based examination of GRAF family member expression in the mouse, however, we noticed that message levels for GRAF2 (the GRAF family member most homologous to GRAF3) were qualitatively higher than GRAF3 in nearly all SMC-containing

[3] This chapter contains text, data, and figures being prepared for publication as an original research article and is co-authored by Rachel A. Dee, Xue Bai, Christopher P. Mack, and Joan M. Taylor

tissues. According to the GTEx database, expression of GRAF2 in human SMC tissues is not only higher than GRAF3 (approximately 10 fold in arteries) but surprisingly more SMC-selective (**Figure 3.1**).

GRAF2 (also known as PSGAP or Arhgap10[4]) is the 2nd identified member of the GRAF family of BAR-PH-GAP proteins known to regulate RhoA and (to a lesser degree) CDC42 *in vitro* and *in vivo* [249]. Over expression of GRAF2 in fibroblasts results in reorganization of cytoskeletal structures and changes in cellular morphology. GRAF2 is essential for Proline-rich tyrosine kinase 2 (PTK2) regulation of cytoskeletal organization [249]. Like other members of the GRAF family, GRAF2 interacts with focal adhesion kinase (FAK). Because of all these similarities, we hypothesized that GRAF2 may also play a role in BP and vessel tone regulation like GRAF3 does.

The goal of this study was to explore the function of GRAF2 in SMC and its role in blood pressure homeostasis and arterial stiffening. We have found that global GRAF2 depletion results in significant elevation of BP and an increase in SMC contractile gene expression and collagens associated with CVD. This may explain why smooth muscle-specific knockout mice display an increase in pulse wave velocity (PWV) (data not shown). These results may lead to anti-hypertensive therapies that can achieve benefits that protect against HTN as well as other manifestations of CVD.

[4] sometimes Arhgap10 is also used interchangeably with Arhgap21, especially in the cancer field but GRAF2 and arhgap21 are NOT the same protein. GRAF2 has a Bar, PH, GAP and SH3 domain, while Arhgap21 has a PDZ, PH, and GAP domain.

3.2 Results

3.2.1 GRAF2 is strongly and selectively expressed in SMC

To explore GRAF2's role in regulating vessel tone and blood pressure homoeostasis *in vivo*, we generated a GRAF2-depleted mouse line using a "knockout first" approach (**Figure 3.2**), using ES cells that contained an inhibitory LacZ gene trap within the GRAF2 seventh intron (**Figure 3.2**). Crosses between *GRAF2*$^{+/gt}$ mice yielded offspring in the appropriate Mendelian ratios. Western Blot analysis of bladders from GRAF2$^{gt/gt}$ mice showed a 90+% reduction of GRAF2 protein (**Figure 3.3B**). Inclusion of the LacZ/neo insertion cassette into the GRAF2 transcript results in a heterologous protein that contains GRAF2 amino acids 1-234 fused in frame to the β-Gal reporter gene allowed us to visualize GRAF2 expression in various mouse tissues. As expected, β-Gal staining in gene trap mice closely mimicked endogenous GRAF2 expression with strong staining in the aorta, liver, lung, mesentery, and bladder, and lack of GRAF2 expression in the brain (**Figure 3.3A**). High power images of the lung co-stained with β-Gal and SM-alpha actin antibodies revealed that expression was restricted to SMC.

3.2.2 GRAF2 depletion elevates blood pressure

To test whether GRAF2 also contributed to BP homeostasis we measured basal blood pressures in our *GRAF2*$^{gt/gt}$ mice. Tail cuff measurements revealed an 18 mmHg increase in basal systolic BP and similar increases in diastolic pressure and MAP (13 mmHg and 15 mmHg, respectively; $p<0.05$; n=3 for *Wt*, n=5 for *GRAF2*$^{gt/gt}$) (**Figure 3.4**). There was no significant difference in heart rate between the two groups. To test whether GRAF2 from smooth muscle

cells, specifically, is the cause for this effect, we created tamoxifen-inducible, smooth muscle-specific GRAF2 knockout mice. Starting with the "knockout first" mouse, we crossed them to flippase (Flp) recombinase mice to remove the lacZ cassette, restoring *GRAF2* to normal, but leaving exon 8 flanked by loxP sites. We then crossed these $GRAF2^{flox/flox}$ mice with tamoxifen inducible *SM MHC-CreER*^{T2} mice. Tamoxifen treatment results in a significant decrease of GRAF2 expressed in tissues (**Figure 3.3C**).

2.2.3 GRAF2 expression is increased in mice with HTN

Previously, we've found that GRAF3 expression is increased with HTN in mice as part of a pressure-sensing feedback mechanism to try to keep BP under control. GRAF2 may be involved in a similar feedback mechanism; PCR analysis of aortas from L-NAME treated mice and renin transgenic hypertensive mice showed significantly higher amounts of GRAF2 compared to untreated, WT mice (**Figure 3.5E**).

3.2.4 GRAF2 depletion causes an increase in expression of SMC contractile genes and collagens, increasing vessel stiffness

Similar to what we have observed with GRAF3, ectopic expression of GRAF2 markedly attenuated RhoA-dependent actin stress fiber and focal adhesion formation in SMC (**Figure 3.5A**). Conversely, siRNA-mediated depletion of GRAF2 in human AoSMC led to a significantly higher expression SMC contractile genes such as myosin heavy chain (MHC), smooth muscle alpha actin (SMA), and calponin (CNN) (**Figure 3.5B** and **3.5C**). GRAF2 depletion in rat aortic SMC also led to an upregulation in MHC, SMA, and CNN, albeit much less dramatically. Interestingly, siRNA knockdown of GRAF2 in RaAoSMC also dramatically

augmented synthesis of collagens Col3a1, Col4a1, and Col8a1 (**Figure 3.5D**). These collagens were also significantly augmented in bladders isolated from *GRAF2*$^{fl/fl}$ *SM MHC-CreER*^{T2} mice 9 days after being treated with tamoxifen (**Figure 3.5F**). This a highly significant finding given the fact that the collagen to elastin ratio is a major determinant of arterial compliance and because polymorphisms in Col4a1 and Col8a1 have been shown to impacted pulse wave velocity (PWV) and because monogenic mutations in Col3a1 and Col4a1 have been associated with aneurisms[250]. Furthermore, we found that PWV of *GRAF2*$^{gt/gt}$ mice was significantly elevated (**Figure 3.5G**). Thus, GRAF2 regulates SMC contractility and vessel stiffness.

3.3 Discussion

Decreased vessel compliance is currently used as a predictive biomarker of ageing and CVD[251-253]. Under normal conditions, pulse pressure expands the arteries and transfers energy to the arterial wall, resulting in a deceleration of pulse velocity as blood travels the body. When arteries have stiffened however, they cannot expand as much and cannot accept as much energy transfer. This excess energy is unfortunately unloaded onto the vessels of the microcirculation, leading to end organ damage[254], therefore increased PWV has been associated with a greater risk of CVD, independent of BP[255]. Because GRAF2 is abundantly and selectively expressed in human SMC and plays a non-redundant role in BP regulation, it is important to further assess the relative SMC-specific contributions of GRAF 2 to vessel tone and BP homeostasis.

We and others have shown that RhoA activation promotes a robust positive transcriptional feedback circuit that activates a cellular “stiffness” gene program that includes integrin and RhoGTPase signaling molecules, cytoskeletal and contractile proteins as well as many of the transcription factors that drive expression of these genes (e.g. SRF and the

Myocardin factors)[256]. Based upon our current findings, we hypothesize that SMC-selective expression of GRAF2 limits vessel stiffening by dampening this robust feed-forward signaling-transcription circuit. As noted above siRNA-mediated depletion of GRAF2 in cultured SMC induced a number of changes that enhance intrinsic cellular stiffness including an increase in the size and number of focal adhesions and increases in the expression of SMMHC, SM α-actin, and calponin. Moreover, since we have shown that GRAF2-depletion promoted gene expression programs in SMC that increase both cellular and extracellular matrix (ECM) stiffness, we hypothesize that GRAF2, like GRAF3, act to limit age-dependent arterial stiffness and its detrimental effects on cardiovascular function.

Our findings that homozygous GRAF2 gene trap mice are hypertensive (+18 mm Hg) and that collagen expression was upregulated in GRAF2-deficient cells and the GRAF2 SM-specific mice within just 1 week following tamoxifen treatment provides strong support for our postulate that SMC-GRAF2 depletion will induce HTN and aberrant vascular stiffness. Thus, we are poised to better understand the contributions of vascular stiffness to vessel remodeling, rarefaction, and target organ damage and our results will have important implications for targeting of anti-hypertensive therapy to achieve benefits beyond lowering BP.

3.4 Materials and Methods

Generation and characterization of GRAF2 depleted mice

GRAF2 "knockout-first" gene trap ES cells were developed and distributed by the European Mouse Mutant Cell Repository (EuMMCR; Munich, Germany). Chimeric mice were produced in-house by injection of *GRAF2*$^{+/gt}$ ES cells into the blastocoel cavity of mouse

blastocysts by standard procedures. The *GRAF2*^{gt} strain was established using # independent chimeras that demonstrated germline transmission when bred to wild-type C57bl6 mice. Homozygous *GRAF2*$^{gt/gt}$ mice were generated by crossing F1 *GRAF2*$^{gt/+}$ mice. GRAF2$^{fl/fl}$ mice were generated by crossing *GRAF2*$^{gt/gt}$ mice to flippase (Flp) mice. Tamoxifen inducible SM-specific knockout mice were created by crossing *GRAF2*$^{fl/fl}$ females to *SM MHC-CreER*^{T2} males. All experiments were performed using age, sex and littermate genetic controls. Animal husbandry was provided by staff within the University of North Carolina Division of Comparative Medicine and all animal procedures were approved by our accredited American Association for Accreditation of Laboratory Animal Care committee and the Institutional Animal Care and Use Committee.

LacZ tissue staining

Tissues from GRAF2 mice were processed for LacZ staining using previously published methods[257]. Briefly, tissues were rinsed in 1 × phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature. After three 10-min washes in wash buffer (0.1 M phosphate buffer (pH 7.3), 0.1% sodium deoxycholate, 0.02% NP-40, 0.05% bovine serum albumin (Sigma)), tissues were incubated 30 min to overnight at RT in X-Gal staining solution (wash buffer containing 1 mg of X-Gal ml^{-1} (Sigma), 5 mM ferrocyanide and 5 mM ferricyanide). Tissues were then post fixed in the appropriate fixative, cleared and imaged on a Leica WILD Macroscope M420.

Western blotting

Cells or tissues were lysed in RIPA buffer + 0.5% Triton with protease and phosphatase inhibitors. Protein concentration was determined by using a colorimetric BCA assay (Pierce). Lysates were electrophoresed on 10-15% SDS-PAGE gels, transferred to PVDF membrane and immunoblotted with specific antibodies as indicated using a 1:1000 dilution. GRAF2 antibody was from Proteintech; myosin heavy chain (MHC), smooth muscle alpha actin (SMA), and calponin (CNN) antibodies were from Abcam; GAPDH antibody was from Novus Biologicals; alpha-tubulin antibody was from Sigma. Blots were washed in TBST (TBS plus 0.05% Triton X), followed by incubation with horseradish peroxidase conjugated secondary antibodies (Amersham) at a 1:2,000 dilution. Blots were visualized after incubation with chemiluminscence reagents (ECL, Amersham).

Blood Pressure Measurements

Conscious blood pressure was measured in male mice aged 12–16 weeks using a tail cuff detection system (CODA High Throughput System, Kent Scientific). Briefly, mice were restrained in CODA animal holders and placed on a warming platform, heated to 38°C. Once tails reached 32°C, cuffs were placed on tails. Mice were subjected to 30 measurements over a period of 30 min taken every day (between 08:00 and 10:00) for 10 consecutive days as previously described[258]. Data are presented as an average of measurements on the final 3 days.

Cell culture and Immunofluorescent Staining

HuAoSMC were purchased from Lonza and maintained in smooth muscle growth medium-2 (SmGM-2) supplemented with growth factors and 5% FBS. Rat aortic smooth muscle cells

(RaAoSMC) were maintained in DMEM-F12 media (Gibco), supplemented with 10% fetal bovine serum and 0.5% penicillin/streptomycin. Cells were transfected with HA-GRAF2 plasmids using Trans-IT (Mirus Bio) transfection reagents according to manufacturer's protocol. After 24 hours, cells were permeabilized, blocked and incubated with primary mouse anti-HA antibody overnight (Cell Signaling). After washing with PBS, slides were incubated for 1 hour with Alexa Fluor488-Goat anti mouse antibody or 555-conjugated phalloidin (Invitrogen). Cells were imaged at 40X on a Zeiss 700 Confocal Laser Scanning Microscope.

siRNA knockdowns

The following siRNAs were obtained from Invitrogen: nontargeted control (NTC) siRNA (to GFP) 5′-GGUGCGCUCCUGGACGUAGCC-3′, GRAF2 5′- [dT][dT]-3′ (sense) and 5′- [dT][dT]-3′ (antisense). GRAF3 5′- [dT][dT]-3′ (sense) and 5′- [dT][dT]-3′ (antisense). GRAF2 and GRAF3 siRNA was obtained as a sense/antisense mixture. HuAoSMCs were transfected with 50 nM GRAF2 or NTC siRNA using Dharmafect siRNA transfection reagent (Dharmacon). HuAoSMCs were harvested 72 hours after knockdown.

Quantitative PCR

RNA was isolated from cells or tissues using RNeasy Mini Kit (Qiagen). RNA was treated with DNase (Qiagen) to eliminate contaminating genomic DNA. RNA underwent first-strand cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). Twenty nanograms cDNA was used in quantitative or semiquantitative PCR assays. Semiquantitative PCR primers used were: Real-time PCR primers used were: ; GAPDH, 5′-ATGGGTGTGAACCACGAGAA, 3′-GGCATGGACTGTGGTCATGA.

Hypertension models

For L-NAME experiment, mice were treated with L-NAME in drinking water (450 mg/L) for 14 days. Renin Transgenic mice (RTG), developed by Oliver Smithies' Lab, endogenously express less renin and exhibit higher BP. All groups were maintained on standard chow.

Pulse Wave Velocity

Pulse Wave Velocity (PWV) was calculated for 10 month old WT and GRAF2$^{gt/gt}$ mice (n=6 per group). In brief, blood flow was recorded using a 20-MHz pulsed Doppler probe at the levels of the carotid and femoral arteries, and PWV was calculated by dividing the separation distance by the difference in pulse wave arrival time in respect to ECG R-peaks as previously reported[259].

Statistics

All data represent at least 3 individual experiments and are presented as means ± standard deviation. Means were compared by Student's t-test and statistical significance is reported as p-values. Sample sizes were chosen based on an extensive literature search and standard exclusion criterion of two standard deviations from the mean were applied. Band intensities were quantified using ImageJ software. All statistics were calculated in Excel or GraphPad Prism8.

3.5 Figures

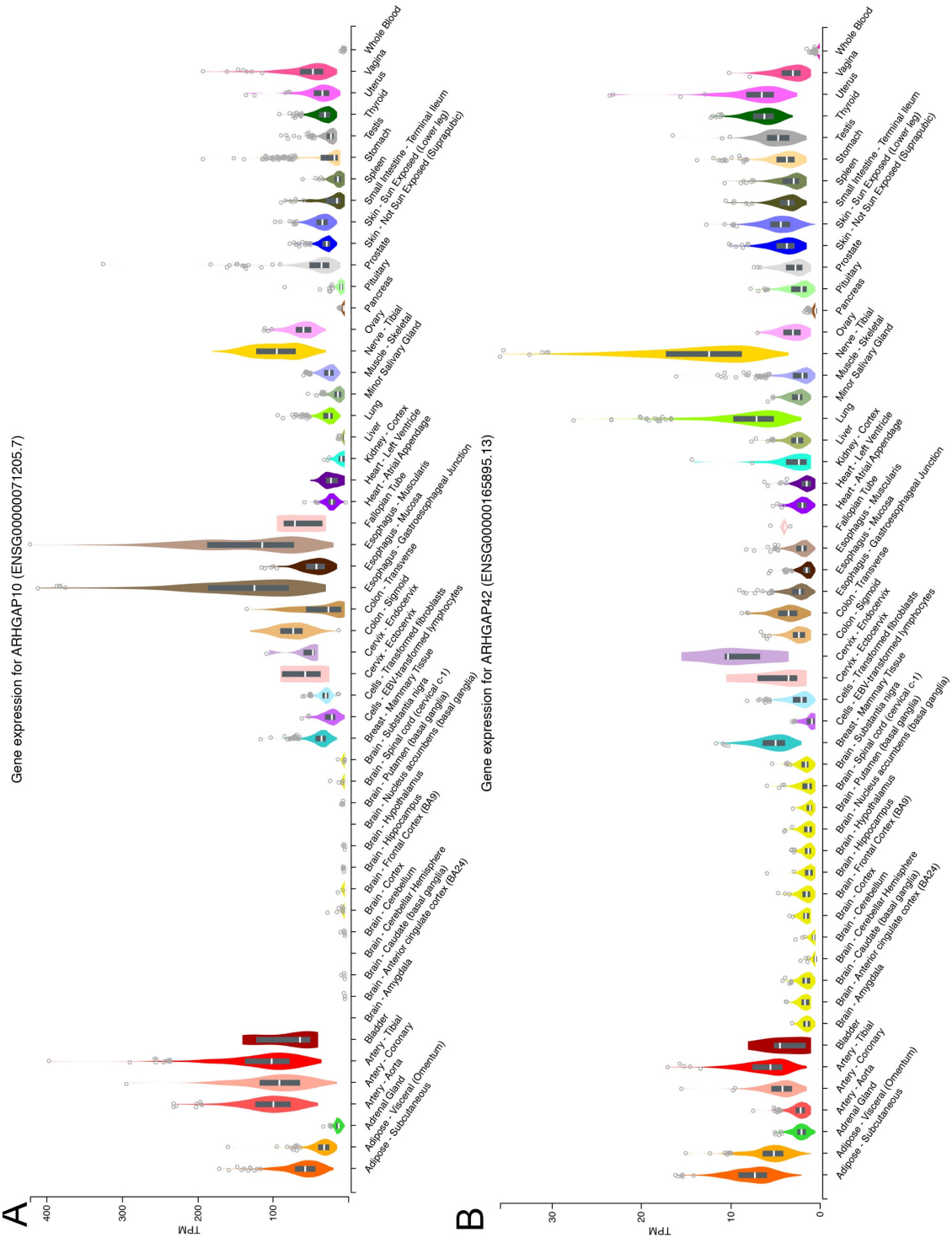

A
Gene expression for ARHGAP10 (ENSG00000071205.7)
TPM
400
300
200
100
B
Gene expression for ARHGAP42 (ENSG00000165895.13)
TPM
30
20
10
0
Whole Blood
Vagina
Uterus
Thyroid
Testis
Stomach
Spleen
Small Intestine - Terminal Ileum
Skin - Sun Exposed (Lower leg)
Skin - Not Sun Exposed (Suprapubic)
Prostate
Pituitary
Pancreas
Ovary
Nerve - Tibial
Muscle - Skeletal
Minor Salivary Gland
Lung
Liver
Kidney - Cortex
Heart - Left Ventricle
Heart - Atrial Appendage
Fallopian Tube
Esophagus - Muscularis
Esophagus - Mucosa
Esophagus - Gastroesophageal Junction
Colon - Transverse
Colon - Sigmoid
Cervix - Endocervix
Cervix - Ectocervix
Cells - Transformed fibroblasts
Cells - EBV-transformed lymphocytes
Breast - Mammary Tissue
Brain - Substantia nigra
Brain - Spinal cord (cervical c-1)
Brain - Putamen (basal ganglia)
Brain - Nucleus accumbens (basal ganglia)
Brain - Hypothalamus
Brain - Hippocampus
Brain - Frontal Cortex (BA9)
Brain - Cortex
Brain - Cerebellum
Brain - Cerebellar Hemisphere
Brain - Caudate (basal ganglia)
Brain - Anterior cingulate cortex (BA24)
Brain - Amygdala
Bladder
Artery - Tibial
Artery - Coronary
Artery - Aorta
Adrenal Gland
Adipose - Visceral (Omentum)
Adipose - Subcutaneous

Figure 3.1 Smooth muscle specificity of GRAF2 and GRAF3 gene expression in humans (previous page). Gene expression of **(A)** GRAF2 (ARHGAP10) and **(B)** GRAF3 (ARHGAP42) in human tissues from GTEx database. Images provided by GTEx consortium (www.gtexportal.org accessed on 7/17/2019).

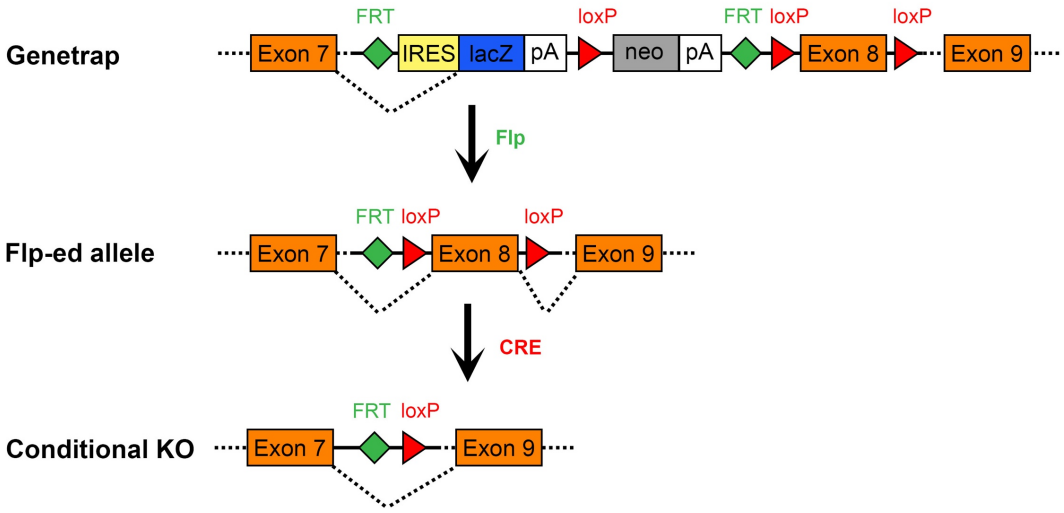


Figure 3.2 Generation of GRAF2 depleted mice. Schematic detailing the "knockout first" strategy for creating genetrap (*GRAF2*$^{gt/gt}$) or conditional knockout (*GRAF2*$^{fl/fl}$) mice. IRES, internal ribosome entry site; lacZ, lacZ reporter; pA, polyA; neo, neomycin cassette.

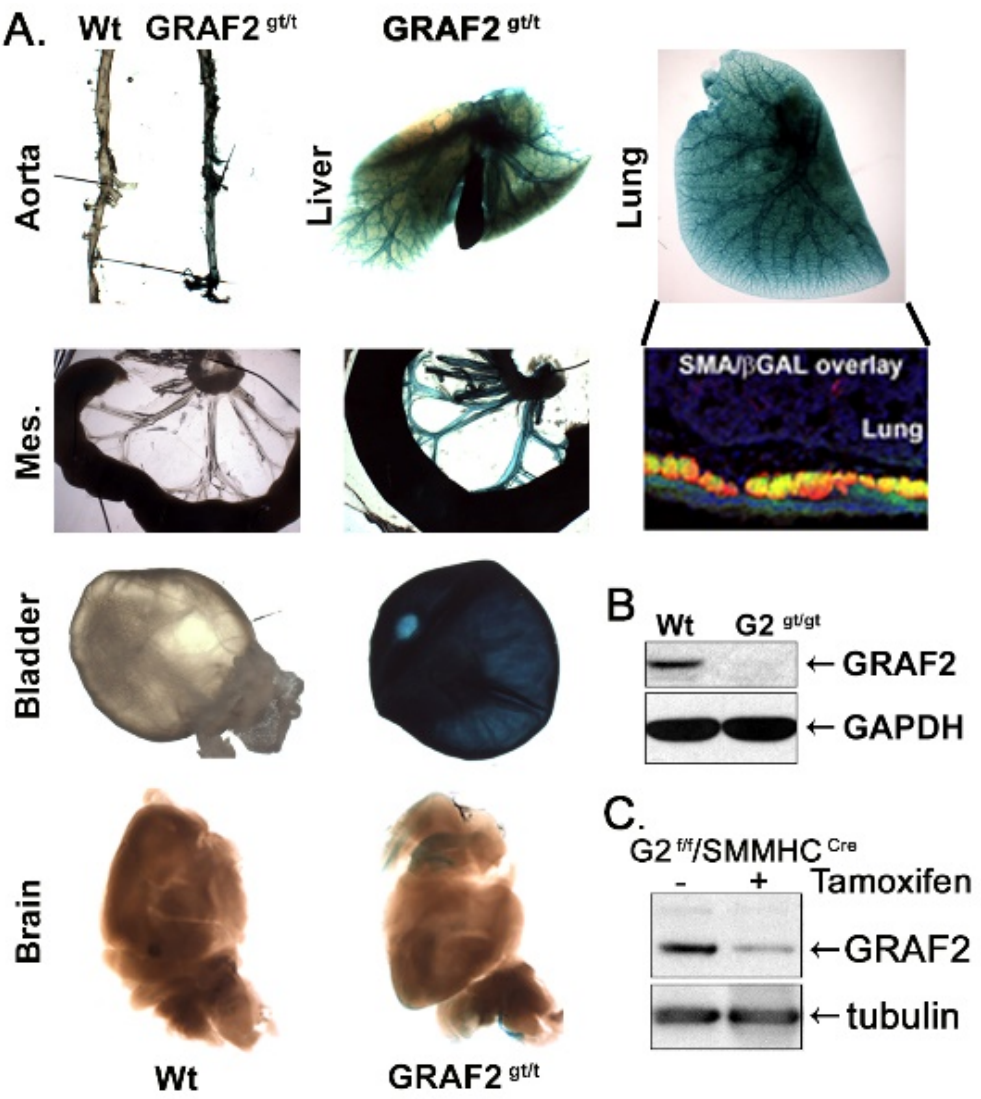


Figure 3.3 Novel GRAF2 deficient mouse model. **(A)** GRAF2 expression pattern revealed by whole mount Lac Z staining (blue) or by dual immunohistochemical staining (in frozen section) for β-GAL (green), SM α-actin (red), (DAPI, blue) from *GRAF2*$^{gt/+}$ mice. No significant LacZ staining or β-GAL immunofluorescence was observed in tissues from Wt mice. Mes., mesenteric. Western analysis of GRAF2 depletion in bladders from **(B)** *GRAF2*$^{gt/gt}$ mice and **(C)** *GRAF2*$^{fl/fl}$ *SM MHC-CreER*^{T2} mice (treated with tamoxifen, 100mg/kg via oral gavage for 3 consecutive days).

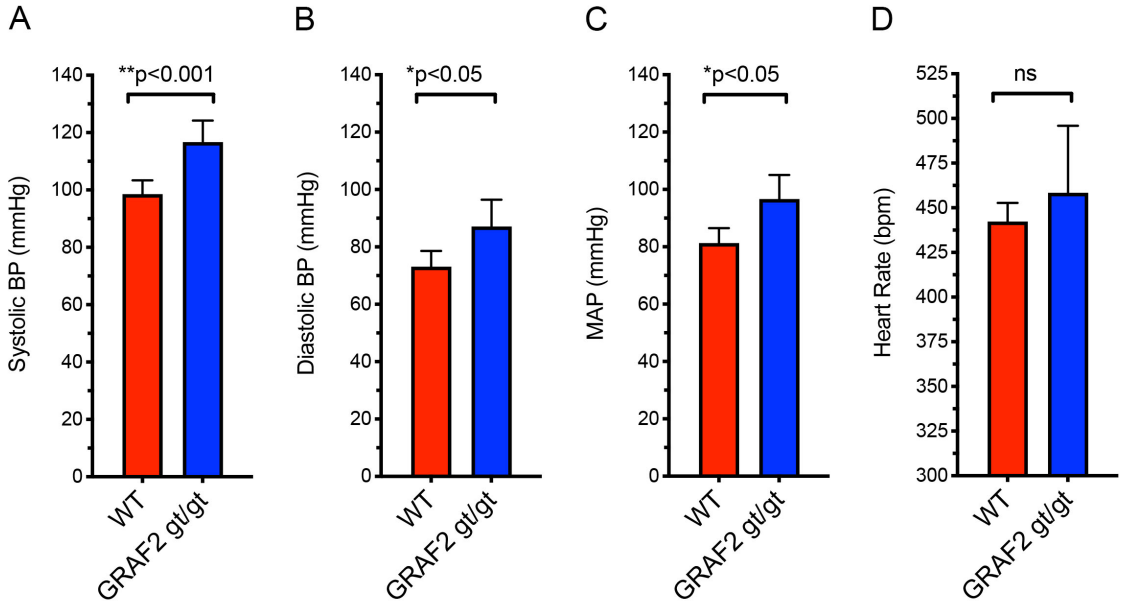


Figure 3.4 GRAF2 deficiency leads to increased basal blood pressure. (A) systolic pressure, **(B)** diastolic pressure, **(C)** mean arterial pressure, and **(D)** heart rate of 12-16 week old male *GRAF2*$^{gt/gt}$ or *WT* mice measured via tail cuff. Data are expressed as mean ± SD; n=3 for *WT* and n=5 for *GRAF2*$^{gt/gt.}$ *p<0.05 vs. WT; **p<0.001 vs. WT (Student's t-test).

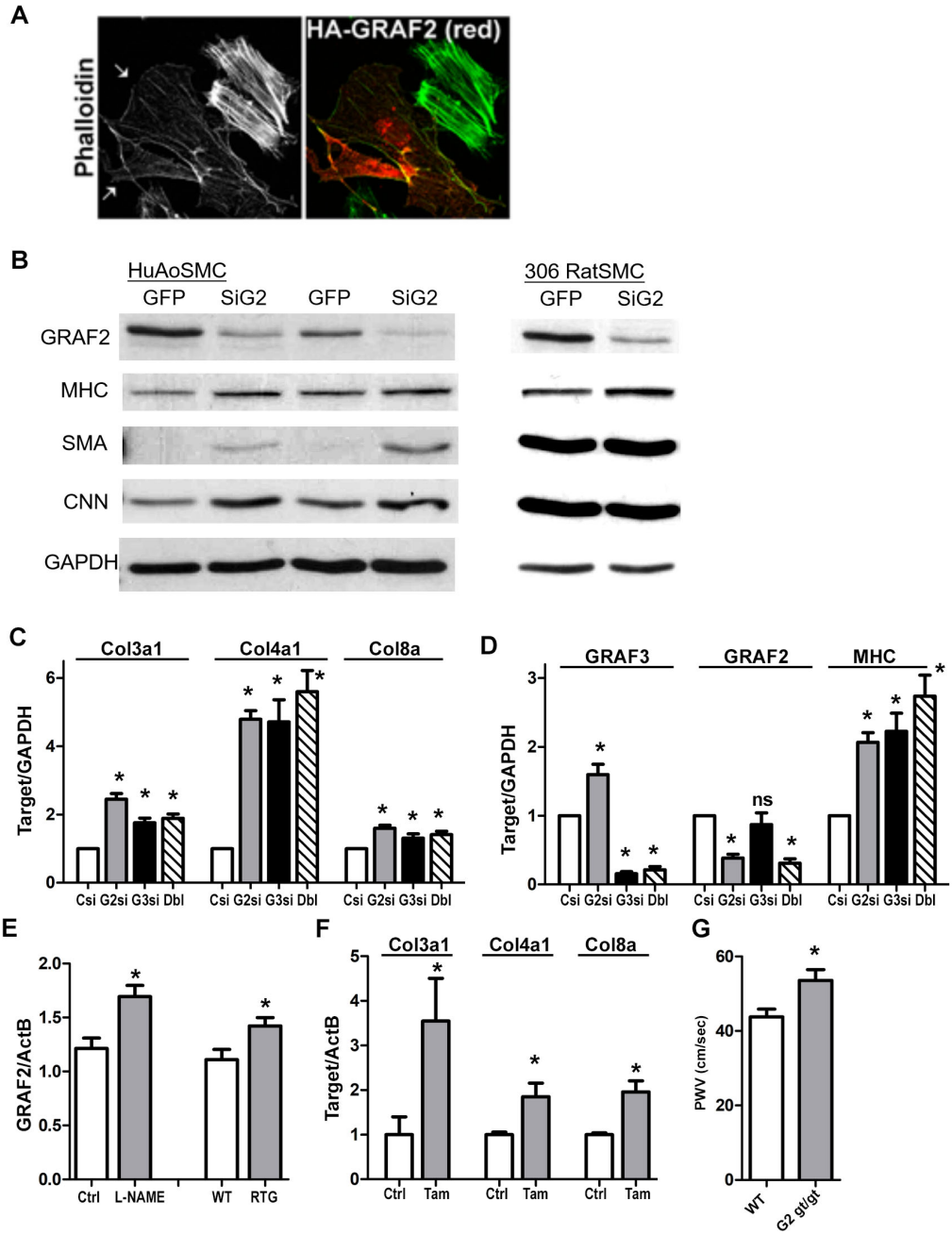


Figure 3.5 GRAF2 regulates SMC phenotypes. **(A)** Expression of HA-GRAF2 (arrow, red) in RaAoSMC markedly attenuated stress fiber formation, while **(B)** depletion by siRNA significantly induced contractile protein levels in HuAoSMC and RaAoSMC. **(C)** and **(D)** RaAoSMC depletion of GRAF2 (and GRAF3) by siRNA significantly increased collagen production. **(E)** Aortas from hypertensive mice treated with L-NAME for 14 days or renin transgenic (RTG) mice show an increase in GRAF2 expression. **(F)** Collagens are significantly increased in aortas from tamoxifen treated *GRAF2*$^{fl/fl}$ *SMMHCCreER*^{T2} mice **(G)** Pulse Wave Velocity of *WT* or *GRAF2*$^{gt/gt}$ mice (n=3, n=5, respectively). *p<0.05 vs. control (Ctrl) or WT (student's t-test)

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Summary of work and future directions

The studies presented in this dissertation reveal the importance of GRAF2 and GRAF3 in blood pressure homeostasis, SMC contractility, and vessel compliance. The data herein reinforce the importance of RhoA signaling in CVD development and strengthen our understanding of the vascular contribution to HTN. The initial purpose of these studies was to assess the therapeutic potential of GRAF3. In Chapter 2, we show for the first time that overexpression of GRAF3 in SMC has the capability to significantly decrease systolic blood pressure in normotensive and hypertensive mice. Furthermore, we discovered that stretch-activation of Src and FAK kinases lead to phosphorylation of GRAF3 at Y376, relieving GRAF3's autoinhibition. Activation of GRAF3 reduces RhoA activity and decreases phosphorylation of MLC in SMC. Recent analysis has shown that GRAF2 is more highly expressed than GRAF3 in human smooth-muscle tissues, so studies in Chapter 3 sought to understand the role of GRAF2 in blood pressure homeostasis. We found that GRAF2 depletion in mice results in significantly increased systolic, diastolic, and mean arterial pressure. Moreover, depletion of GRAF2 leads to an increase in SM contractile gene expression and an increase in collagens. Coupled with the fact that GRAF2 depleted mice have significantly increased pulse wave velocity, this indicates that GRAF2 may play a role in arterial stiffening as well as blood pressure regulation.

While our collective work has made significant contributions to the field, it has also prompted many unanswered questions. Excitingly, we found that once mice overexpressed GRAF3^{RQ}, their blood pressure significantly decreased and were able to consistently stay lower than their non-overexpressing counterparts, even after being subjected to L-NAME induced HTN. Moving forward, it will be necessary to test whether overexpressing GRAF3^{RQ} *after* the induction of HTN would effectively lower BP. This would more accurately mimic hypertensive treatment in humans, where patients seek treatment for HTN after they are diagnosed rather than seek preventative care prior to symptoms. It would also be interesting to test the widespread ability of GRAF3 overexpression to prevent or reverse BP increases in other models of essential HTN, each of which works through a different mechanism and imitates different features found in human HTN[233, 260]. L-NAME treatment prevents dilation by inhibiting nitric oxide synthase. DOCA on the other hand is an aldosterone precursor that imitates the effects of mineralocorticoid and glucocorticoids, recapitulating both volume overload and increased sympathetic neural signaling and AII infusion is part of the renin-angiotensin-aldosterone system that modulates renal sodium handling.

More and more emphasis has been placed on the need to study sex differences in CVD. Although men have higher incidence rates of CVD than age-matched premenopausal women, that pattern is reversed post-menopause[261]. Sex differences relating to CVD have been observed in animals, as well. For example, spontaneously hypertensive rats show age-dependent increases in BP that are greater in males than females[261]. In pharmacologically induced models of HTN, these differences can be as great as 30 or 50 mmHg between males and females[261]. While many studies attribute these differences to hormones, that is not exclusively the case. Therefore, it would be of interest to explore if GRAF3 overexpression has different effects on BP in males

and females. One limitation of our current GRAF3 SM-specific overexpression mouse model is that we are only able to study males; the SMMHC^{CreERT2} line is currently the most specific and robust SMC-specific Cre line available, however, this BAC transgene was randomly incorporated into to the Y chromosome. To this end, there is a SM22-CreER^{T2} line available that will enable SMC-selective recombination in adults in both sexes. It should be noted, however, that there is some concern that this Cre is expressed in myeloid cells in addition to SMC[262], so more work would have to be done to show that the overexpression in SMC is the cause of any seen effects.

Another well-documented and relevant factor in HTN and CVD development is age, but it's estimated that aging is only studied in 4% of all NIH-funded HTN studies[233, 263]. The onset of CVD doesn't usually occur in humans until middle- or old- age. Hypertension studies, including our own, are not always performed at the equivalent later ages in mice. Whereas human middle- and old- age are considered from 38-47 years and 55-69 years respectively, in mice these age groups are considered from 10-14 months and 18-24 months (**Table 4.1**) [264]. It would be of clinical importance therefore, to study the effect of short and long term GRAF3 overexpression in aged mice.

In Chapter 2 we presented a molecular model of GRAF3 that is supported by our experimental findings that the GRAF3 BAR-PH domains autoinhibit GRAF3's GAP domain and that Y376 is in a location accessible for phosphorylation and activation. We built this model from similarly structured Appl1 and GRAF1 because the structure of GRAF3 is currently unsolved. This is because GRAF3 is hard to solubilize and therefore hard to crystalize. But, as crystallization methods advance, we hope to be able to solve the structure of GRAF3 (or at least parts of it) and current collaborations are actively trying this. Having the correct 3D structure of

GRAF3 would give us a better view of "accessibility" and binding pockets for kinases and proteins to interact with GRAF3. It could also be used to make predictions of lead compounds for small molecule activators of GRAF3. The data in this dissertation support the idea that GRAF3 would make a good HTN therapeutic target. In lieu of or alongside crystalizing GRAF3, we can take advantage of small molecule drug screens that are being made more widely available to look for compounds that increase the GAP activity of GRAF3. This strategy is also actively underway. Taking advantage of GRAF3's autoinhibitory mechanism, we can individually validate the compounds by a bioluminescence resonance energy transfer (BRET) assay, where Renilla luciferase is placed on the N-terminus of the BAR domain and fluorescent protein is placed on the C-terminus of the GAP domain. When GRAF3 is in the closed, inactive conformation, the luciferase and the GFP would be in close enough proximity that the luciferase would stimulate the GFP and give off BRET signal. When GRAF3 is in the open, active conformation, the luciferase and GFP would be too far away from one another and no BRET signal would occur. Living cells containing the BRET-GRAF3 construct could be treated with suspected activating compounds and monitored for a decrease in BRET signal.

While the work in this dissertation has focused mainly on the GAP domain activity of GRAF3, it would be of interest to characterize the specific functions of each of the domains within GRAF3 in the context of SMC to expand our portfolio of GRAF3 drug-targeting strategies. BAR domains are known for their ability to sense and form membrane curvature [218] while PH domains bind to phosphatidyl lipids found in biological membranes and help recruit proteins to specific membranes, mediating appropriate cellular compartmentalization or signal transduction interactions[265]. In fibroblasts, the GRAF3 PH domain controlled the localization of GRAF3 at focal adhesions along the cell periphery[208], but this has not been validated in SMC.

SH3 domains modulate protein-protein interactions by binding with moderate affinity and selectivity to proline-rich ligands[266]. SH3 domains have even been known to autoinhibit the membrane-binding capabilities of N-Bar and F-Bar domains in the endocytic protein endophillin and the dynamin-binding protein syndapin[218, 267, 268]. In a study looking at the SH3 domain of Rho family GAPs, they additionally found that the GRAF1 SH3 interacts with PKN3 and PTK2, which play roles in focal adhesion turnover and actin depolymerization[208, 217]. They found that the GRAF2 SH3 domain binds to ASAP2 (recruits paxillin to focal adhesions), TLN1 (component of focal adhesion), and DNAH6 (cytoskeletal motor protein), suggesting that GRAF1 and GRAF2 may play different roles in focal adhesion assembly[217]. They also found that GRAF3 SH3 domain binds to MYH14 (myosin heavy chain), DBN1 (F-actin binding), LRRFIP2 (formin regulatory protein), or FHOD3 (formin actin nucleation and elongation)[217]. Furthermore, the SH3 domain of GRAF1 and GRAF3 have been shown to be necessary for its localization to focal adhesions and actin stress fibers[208]. None of these interactions, however, have been validated in SMC.

Another focus in this dissertation has been to show the importance of the phosphorylation of GRAF3 at Y376, but there are other sites on GRAF3 that are predicted to be post-translationally modified as well (**Figure 4.1C**). For example, S152 is part of a consensus binding site for MAPK, which is known to be activated by G-protein signaling from vasoconstrictive agonists acting on GPCRs on the SMC surface. This serine is part of a serine/lysine/aspartic acid triad that our model predicts would undergo a conformational shift when the serine is phosphorylated (**Figure 4.2A**). This phosphorylation could drive the BAR and PH domains away from one another, causing the GAP domain to "pop up". Our preliminary work shows that SMCs transfected with a phospho-deficient version of GRAF3 (S152A) show

normal stress fiber clearing. Smooth muscle cells transfected with a phospho-mimetic version of GRAF3 (S152D), however, show enhanced stress fiber formation, indicating that GRAF3 phosphorylation at S152 favors the closed, inactive version of GRAF3 (**Figure 4.2B** and **4.3**). Stress fiber formation is further exacerbated when SMC transfected with GRAF3 S152D are also treated with the vasoconstrictor S1P (**Figure 4.2B**). A radioactive kinase assay revealed that p38 delta is a MAPK capable of phosphorylating GRAF3 at S152 (**Figure 4.2C**) and SMCs show decreased phosphorylation of GRAF3 at S152 and decreased pERK signaling when Rho is inhibited pharmacologically (**Figure 4.2D**). This supports the idea of another branch of the RhoA/contraction/GRAF3 feedback loop, in which vasoconstrictive agonists have a more direct effect on GRAF3 than previously thought before (**Figure 4.3**). It would be interesting to not only explore this S152 phosphorylation-activity relationship in more depth using similar studies as those performed in this dissertation, but it would also be interesting to test whether GRAF3 S152 dephosphorylation and concurrent GRAF3 Y376 phosphorylation would result in stronger, more dramatic RhoA inhibition. While Y376 and Y792 are the top hits on PhosphoSite Plus, S152 was not listed at all, despite displaying importance experimentally. This opens the door for the possibility of other important, still unidentified sites of phosphorylation as well.

GRAF2 and GRAF3 share nearly 48% of their amino acid sequence (according to UniProt alignment). This percentage jumps to 65, 56, 62, and 59% when the BAR, PH, GAP, and SH3 domains, respectively, are individually compared. Despite this similarity, PhosphoSitePlus analysis has not identified the same residues on both proteins as the major sites of phosphorylation (**Figure 4.1**). While there seem to be similar regions on GRAF2 and GRAF3 that are both post-translationally modified, Y376 and Y792 stand out in GRAF3 and S591 stands out in GRAF2 as the most-likely sites for phosphorylation. While Y376 and (to some extent,

albeit minimal) Y792 have been explored in this dissertation, no work has been done to validate or explore the significance of S591 in GRAF2 and this would be a good place to start teasing out the mechanism of GRAF2. Our molecular modeling analysis of GRAF3 revealed a site of interest that was not identified by PhosphoSitePlus, so it is possible that there are other residues of importance on GRAF2 besides S591 that could be of interest.

Shown in Chapter 3 and in experiments not shown in this dissertation, we have evidence that GRAF2 and GRAF3 exhibit some degree of compensation. GRAF2 depletion results in compensatory up-regulation of GRAF3 while co-depletion of both GRAFs (*GRAF2*$^{gt/gt}$/*GRAF3*$^{gt/gt}$) led to embryonic lethality. Thus, it has not yet been possible to assess the combinatorial roles of GRAF2 and GRAF3 in the control of BP in adult animals. We are currently in the process of generating *GRAF2/3*$^{fl/fl}$*SMMHCCreER*^{T2} mice which will allow us to knockout both GRAFs in a SMC-specific- and tamoxifen-dependent manner and assess BP and vessel tone effects.

HTN and vascular stiffness are currently caught in a "chicken or the egg" debate. For a long time it has been thought that HTN precedes aortic stiffening, but this paradigm has been called into question recently[269]. Five different animal models of HTN or vascular disorder showed that increased aortic stiffness preceded increased BP and this timing reversal is also supported in humans by the Framingham study[270-275]. Thus far we have found that GRAF2 depleted mice have increased collagen expression and increased PWV, indicating that GRAF2 may be involved in regulation of vessel compliance, but more thorough analysis is needed. To parse out the role of GRAF2 in the timing of stiffness vs HTN, we can measure BP and PWV at different time points in our *GRAF2*$^{gt/gt}$ mice. Because these mice are deficient from birth, there is a chance we could see no significant progression of stiffness overtime. In this case, we can

monitor BP and PWV in *GRAF2$^{fl/fl}$ SMMHC-CreER^{T2}* mice before and multiple timepoints after tamoxifen treatment. Furthermore, we can stain artery sections from these different timepoints with collagen and elastin markers and check for expression of matrix metalloproteinases, which contribute to vascular stiffness through extracellular matrix remodeling.

Shortly after our initial discovery that GRAF2 is more highly expressed than GRAF3 in SM tissues in humans, we noticed the expression of splice variants in these SM tissues (**Figure 4.4**). Whereas GRAF3 has 1 clear dominant isoform, GRAF2 has 3 prevailing isoforms—1 dominant and 2 less dominant, that are missing exons1-9, 21, and 23. These 2 less dominant isoforms are missing the BAR domain of GRAF2, but have fully intact PH and GAP domains. It would be interesting to test how active/functional the less dominant isoforms are and to test if similar GRAF2 isoforms exist in mice.

4.2 Concluding remarks

Hypertension is known as the silent killer because it has no obvious symptoms most of the time. It's the most common chronic disease in Americans, yet HTN still proves to be challenging to treat. Overall, the studies in this dissertation conclude that GRAF2 and GRAF3 are important regulators of blood pressure homeostasis, SMC contractility, vessel tone, and vessel compliance. These studies support the GRAFs as an essential component in a pressure-sensing feedback loop within smooth muscles. With the aid of further investigations, this work could lead to the development of novel antihypertensive treatments that dually function as anti-arteriosclerosis therapies.

4.4 Figures

	Age Range		
	Mature Adult	**Middle Age**	**Old Age**
Mouse	3-6 months	10-14 months	18-24 months
Human	20-30 years	38-47 years	55-69 years

Table 4.1 Comparison between Mouse and Human Ages. Data adapted from Flurkey, Currer, and Harrison, 2007. "The mouse in biomedical research" in James G. Fox (ed.), American College of Laboratory Animal Medicine series (Elsevier, AP: Amsterdam; Boston).

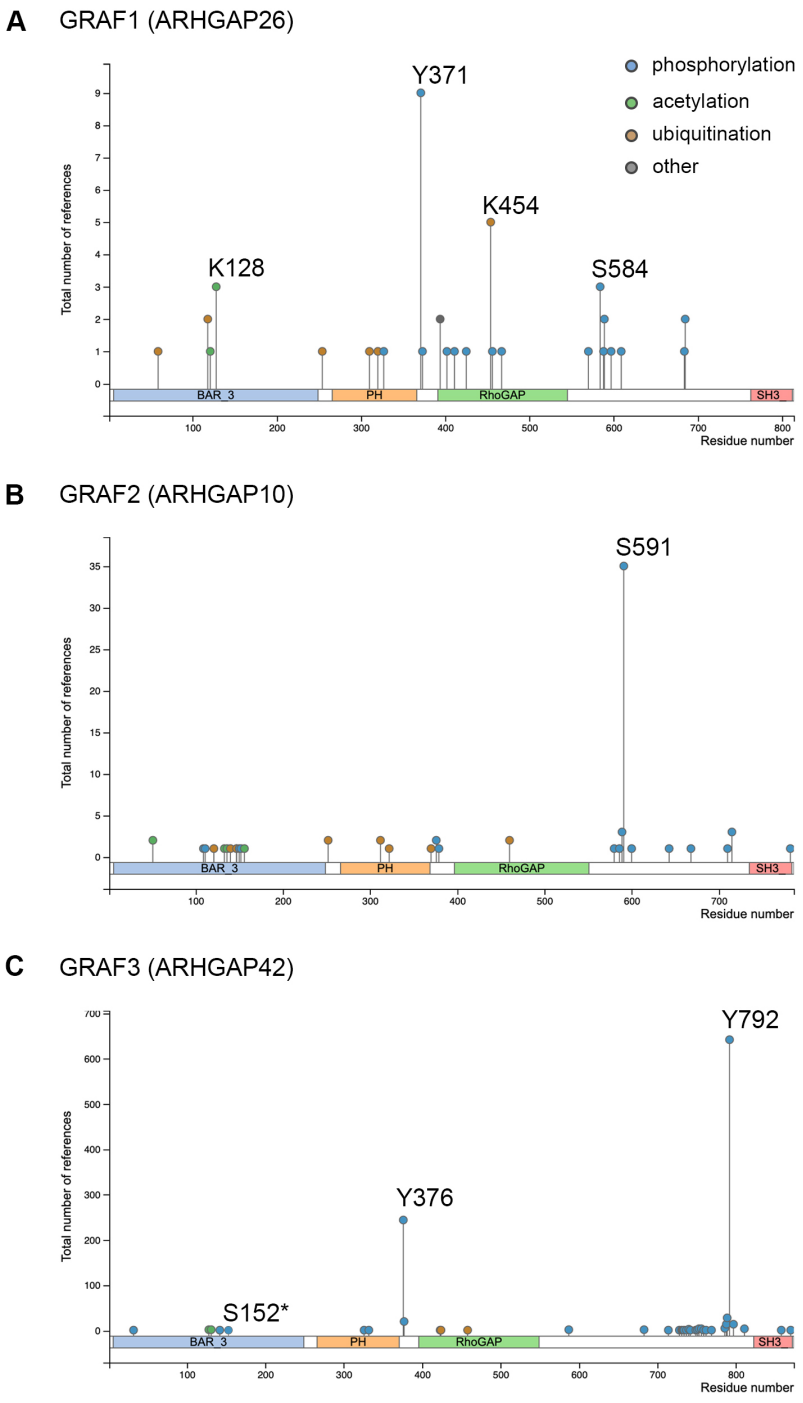


Figure 4.1 Post translational modifications of the GRAF family. Possible sites of phosphorylation (blue), acetylation (green), ubiquitination (tan), or other (gray) post translational modifications on (**A**) GRAF1, (**B**) GRAF2, and (**C**) GRAF3, identified by mass-spectrometry experiments on the PhosphoSitePlus® database. Image modified from PhosphoSitePlus® (www.phoshposite.org accessed on 7/17/19). *S152 bubble has been manually added.

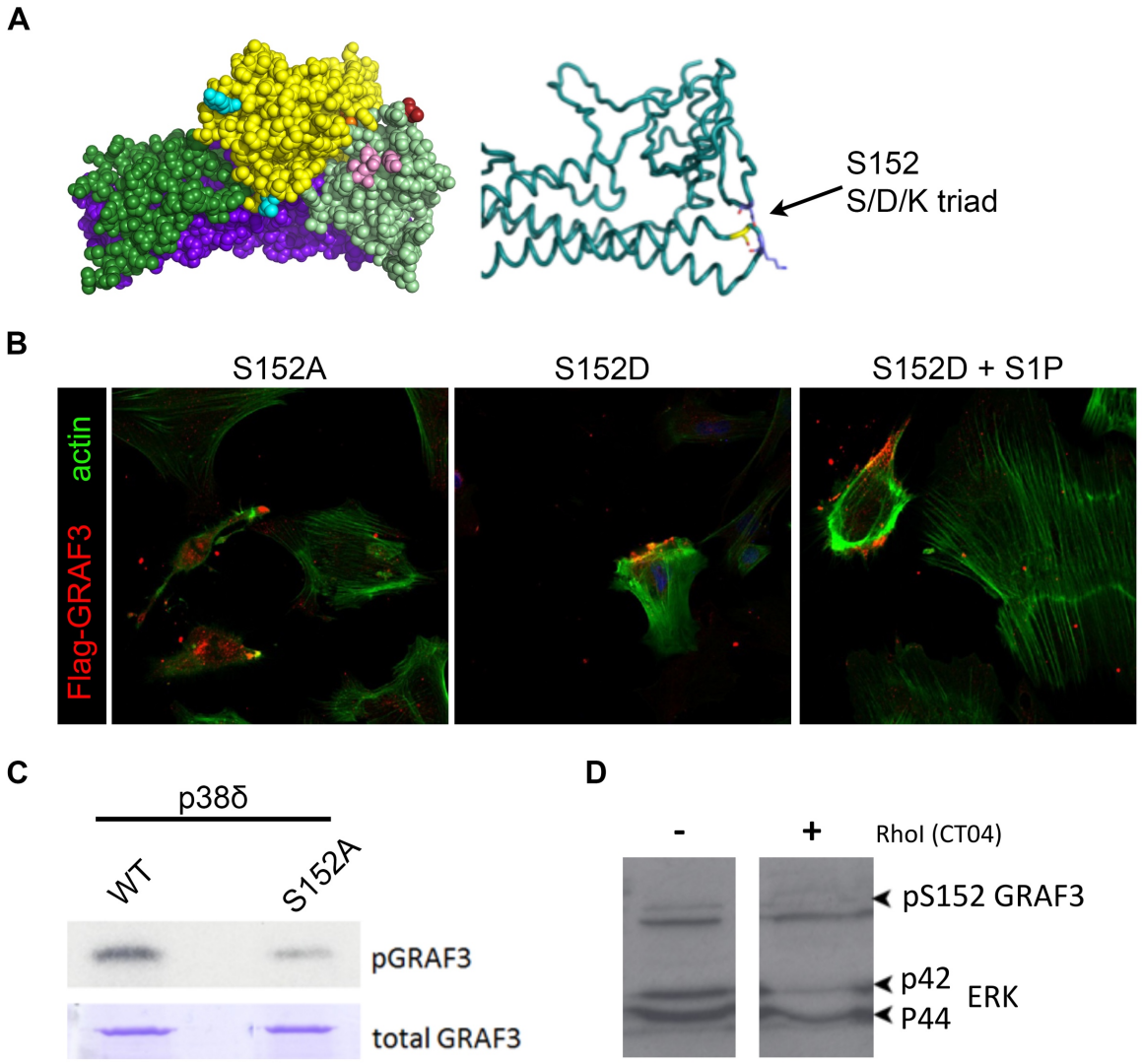


Figure 4.2 Phosphorylation of GRAF3 at S152 by p38 delta affects RhoA signaling. (**A**) Spherical (right) and ribbon (left) 3D models showing the same ½ of GRAF3. Color scheme follows: BAR 1 (dark purple), BAR 2 (dark green), PH 2 (light green), GAP 2 (yellow), RhoA docking sites (pink), C-terminus of PH domain (red), N-terminus of GAP domain (orange). Arrow indicates S152, which is part of a conserved serine(S)/aspartic acid (D)/lysine (K) triad. (**B**) When RaAoSMC are transfected with a phospho-deficient mutant (S152A), cells exhibit normal stress fiber clearing. Cells transfected with a phospho-mimetic mutant (S152D) exhibit enhanced stress fiber formation. Stress fiber formation is further enhanced when these cells are treated with S1P. (**C**) Radioactive Kinase Assay of WT or S152A isolated GRAF3 BAR-PH incubated with active p38 delta. Radiograph, top and Coomassie stain, bottom. (**D**). RaAoSMC were treated with or without CT04, a Rho inhibitor. Western analysis shows decreases in GRAF3 pS152 and pERK signaling when Rho is inhibited.

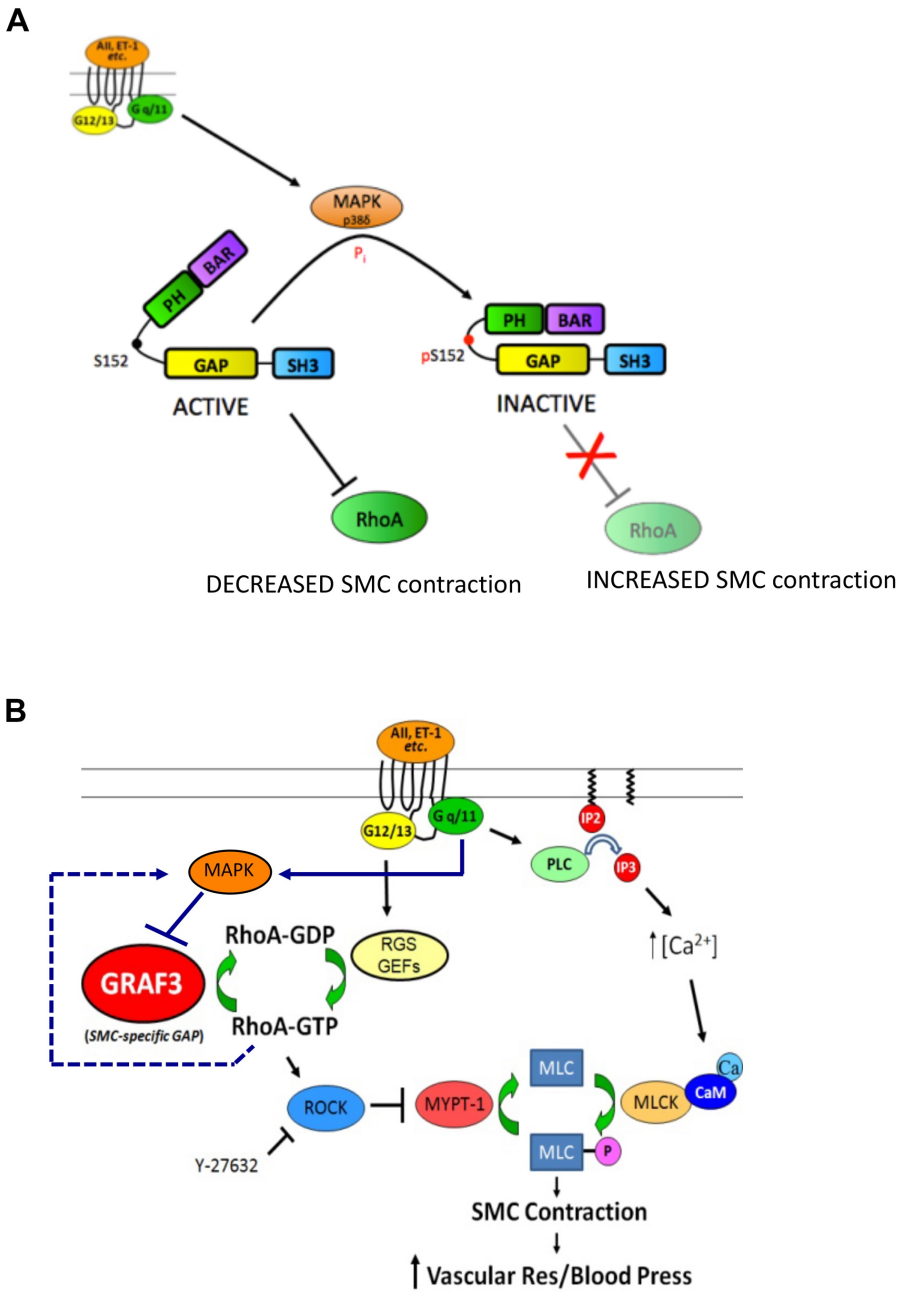


Figure 4.3 Working model of GRAF3 phosphorylation at S152. Vasoconstrictive signals act on GPCRs along the cell membrane of SMCs to promote G-protein signaling which activates members of the MAPK family, such as p38 delta. (**A**) P38 delta phosphorylates GRAF3 at S152. This phosphorylation causes a conformational shift and deactivation of GRAF3, which enhances RhoA signaling and results in increased SMC contraction. (**B**) It is thought that active RhoA feeds back into this signaling pathway to further activate MAPK and enhance contraction.

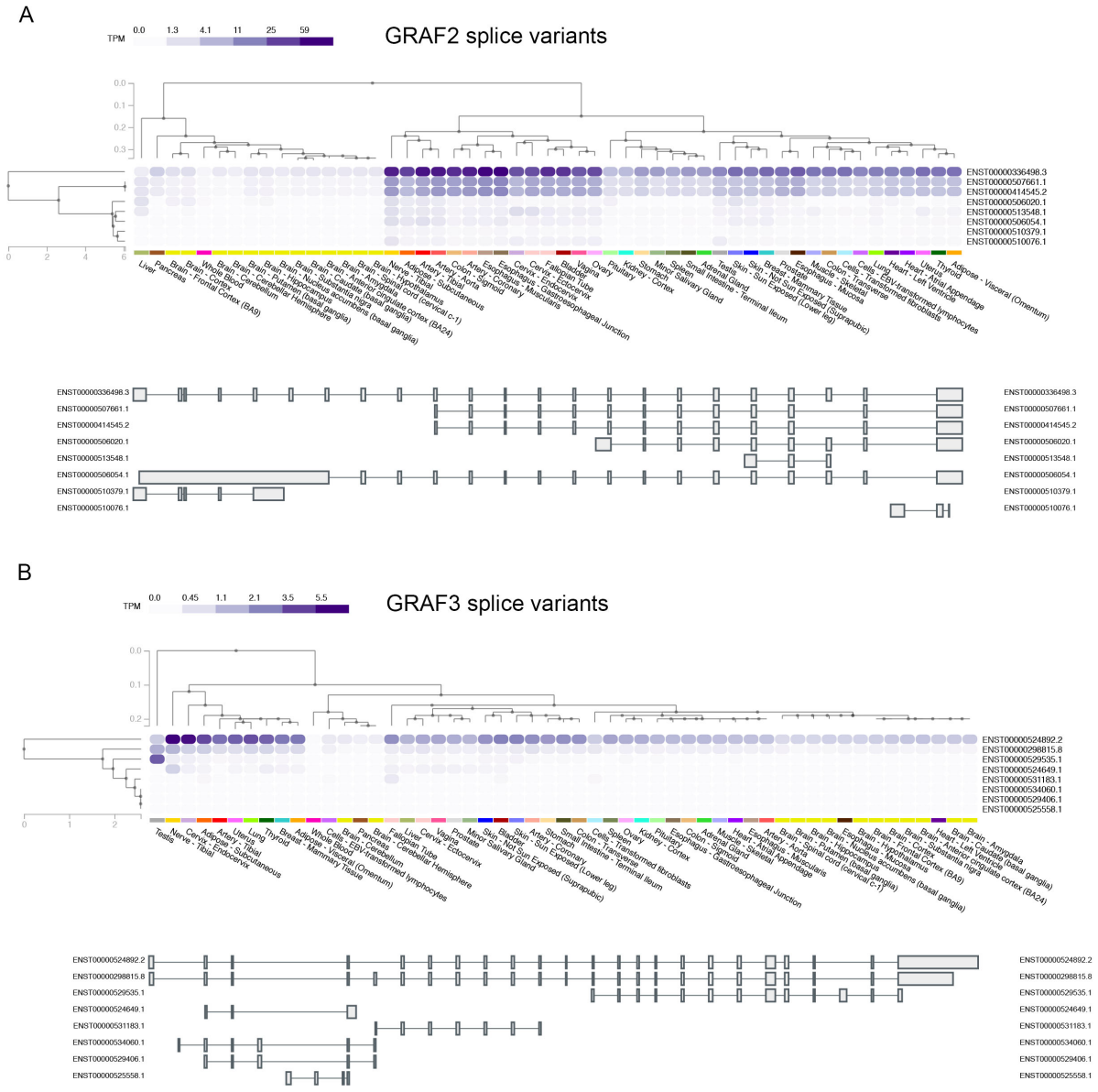


Figure 4.4 GRAF2 and GRAF3 splice variant expression among human tissues. Splice variant expression among different human tissues for **(A)** GRAF2 and **(B)** GRAF3. Images generated from the GTEx database (www.gtexportal.org accessed on 7/17/19).

APPENDIX A: ANALYSIS OF GRAF3 GENOTYPE AND BP IN HUMAN POPULATIONS

rs604723 Genotype	C/C	C/T	T/T	
Systolic BP (mmHg)	130.3	130.2	126.3	
Diastolic BP (mmHg)	81.8	81.1	76.9[A]	
rs604723 Genotype	**C/C**	**C/T**	**T/T**	**MAF**
Mixed European descent	147	108	19	26.6%
African American	54	8	0	6.5%[B]
Other	5	8	1	35.7%[B]
rs604723 Genotype	**C/C**	**C/T**	**T/T**	**MAF**
HTN (%)	141 (69)	78 (64)	12 (60)	22.1%
Non-HTN (%)	64 (31)	44 (36)	8 (40)	26.1%

A group of 346 borderline hypertensive patients (see Appendix B for more detailed characteristics of this group) were genotyped as the rs604723 variation using a TaqMan-based allelic discrimination assay. The resulting genotypes were then correlated with repeated office BP measurements or HTN status (i.e. greater or less than 140 mmHg) or grouped by race. [A]$p<0.05$ vs. diastolic BP measured in patients homozygous for the major allele (C/C); [B]$p<0.001$ vs. MAP in patients of mixed European descent; χ^2 test.

APPENDIX B: TABLE OF CLINICAL COHORT CHARACTERISTICS

Characteristic	Mean ± SD or percent
Age, mean (yrs)	48 ± 12
Age category, %	
30-44 yrs	44
45-64 yrs	45
65+ yrs	11
% female	53
Race, %	
White	77
Black	19
Other	4
Body mass index, mean (kg/m^2)	29 ± 6
Body mass index category, %	
Normal	27
Overweight	36
Obese	38
Total cholesterol (mg/dl), mean	200 ± 38
Current smoker, %	7
Office systolic BP (mm Hg), mean	130 ± 13
Office diastolic BP (mm Hg), mean	81 ± 9
Clinic hypertension, %	29

SD, standard deviation

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